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RESEARCH PAPER

Metformin produces anxiolytic-like effects in rats by facilitating $GABA_A$ receptor trafficking to membrane

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BACKGROUND AND PURPOSE

Altered function or expression of $GABA_A$ receptors contributes to anxiety disorders. Benzodiazepines are widely prescribed for the treatment of anxiety. However, the long-term use of benzodiazepines increases the risk of developing drug dependence and tolerance. Thus, it is urgent to explore new therapeutic approaches. Metformin is widely used to treat Type 2 diabetes and other metabolic syndromes. However, the role of metformin in psychiatric disorders, especially anxiety, remains largely unknown.

EXPERIMENTAL APPROACH

We examined the effects of metformin on anxiety-like behaviour of rats in open field test and elevated plus maze test. We also observed the effect of metformin (10 μM, *in vitro*; 100 $mg \cdot kg^{-1}$, *in vivo*) on the trafficking of $GABA_A$ receptors, as mechanisms underlying the anxiolytic effects of metformin.

KEY RESULTS

Metformin (100 $mg \cdot kg^{-1}$, i.p. 30 min) displayed a robust and rapid anxiolytic effect, without tolerance. Metformin up-regulated the surface expression of $GABA_A$ receptors and increased miniature inhibitory postsynaptic currents (mIPSCs). AMP-activated protein kinase (AMPK) activated by metformin-induced stimulation of forkhead box O3a (FoxO3a) transcriptional activity, followed by increased expression of $GABA_A$ receptor-associated protein (GABARAP) and its binding to $GABA_A$ receptors finally resulted in the membrane insertion of $GABA_A$ receptors.

CONCLUSIONS AND IMPLICATIONS

Metformin increased mIPSCs by up-regulating the membrane insertion of $GABA_A$ receptors, via a pathway involving AMPK, FoxO3a, and the $GABA_A$ receptor-associated protein. Thus metformin has a potential new use in the treatment of anxiety disorders.

Abbreviations

AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation; CS, conditioned stimulus; EPM, elevated plus maze test; FoxO3a, forkhead box O3a; GABARAP, $GABA_A$ receptor-associated protein; mIPSCs, miniature inhibitory postsynaptic currents; OFT, open field test; shRNA AMPKα_2, short hairpin RNA AMPKα_2; shRNA-FoxO3a, short hairpin RNA FoxO3a; US, unconditioned stimulus

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Introduction

Anxiety disorders are among the most common psychiatric conditions and affect millions of people of different ages, races, religions and incomes. However, the molecular basis for the development of the disorders remains largely unknown (Liu *et al.*, 2007; Tasan *et al.*, 2011). Increasing evidence implicates that reduced brain concentrations of the inhibitory neurotransmitter **GABA** and altered function or reduced expression of its principal receptor, the $GABA_A$ receptor, contribute to anxiety disorders (Luscher *et al.*, 2011a; Smith and Rudolph, 2012). Benzodiazepines and other drugs that bind to and modulate the activity of $GABA_A$ receptors are the most commonly used drugs for acute anxiolysis. However, the long-term use of benzodiazepines increases the risk of developing drug dependence and tolerance (Buller and Legrand, 2001; Jacob *et al.*, 2012). Therefore, the medical need for treatments of greater efficacy and decreased tolerance remains high. The accumulation of $GABA_A$ receptors on the neuronal membrane is a critical factor in determining synaptic inhibition (Luscher *et al.*, 2011a). However, few pharmacological approaches that seek to modulate the trafficking of $GABA_A$ receptors to the plasma membrane have been investigated and employed in the treatment of anxiety.

The $GABA_A$ receptor-associated protein (GABARAP) represents the first isolated protein interacting with $GABA_A$ receptors and, accordingly, has received considerable attention (Everitt *et al.*, 2004; Chen *et al.*, 2005). Endogenous GABARAP contributes to the regulation of $GABA_A$ receptor trafficking (Leil *et al.*, 2004). Increasing evidence suggests that the forkhead transcription factor 3 (FoxO3a) directly binds to the promoter region and induces the expression of many autophagy-related genes, including GABARAP (Mammucari *et al.*, 2007). In a variety of analysed cells and tissues, the AMP-activated protein kinase (**AMPK**)-FoxO3a axis plays a central role as a modulator of fundamental processes such as stress resistance, autophagy and antitumour therapy (Greer *et al.*, 2007; Chiacchiera and Simone, 2010; Sato *et al.*, 2012). However, few reports of this axis have focused on the CNS.

Metformin, which is widely used to treat Type 2 diabetes and other metabolic syndromes, exerts potent antihyperglycaemic activity by increasing glucose uptake and decreasing hepatic gluconeogenesis (Zhou *et al.*, 2001). Recent studies have shown that metformin has various biological functions other than its antidiabetic effects, partly through activation of the AMPK signalling pathway (Gantois *et al.*, 2017; Tunc-Ozcan *et al.*, 2017). Intriguingly, in endothelial cells, metformin reduces the levels of intracellular ROS *via* the AMPK-FoxO3a pathway (Hou *et al.*, 2010). Other results have shown that a large proportion of diabetic patients also display symptoms of anxiety (22–48%), which may affect the quality of life in diabetic patients (Edwards and Mezuk, 2012; Lewko *et al.*, 2012; Tovilla-Zarate *et al.*, 2012). Recent studies have reported that metformin improves anxiety-like behaviours secondary to nicotine withdrawal (Brynildsen *et al.*, 2018), transient forebrain ischaemia and diabetes (Sarkaki *et al.*, 2015). However, the effect of metformin on psychiatric disorders, especially anxiety, is poorly understood.

Although there are many brain regions involved in the modulation of anxiety, such as the amygdala, pre-frontal cortex and hippocampus, several lines of evidence indicate that the hippocampal CA1 region is critical for pathogenesis of anxiety-related behaviour in the rodent model (Adams and van den Buuse, 2011; Modol *et al.*, 2011; Kim *et al.*, 2012; Zarrindast *et al.*, 2012; Wells *et al.*, 2013; Naseri *et al.*, 2014; Jimenez *et al.*, 2018). Because metformin by activating AMPK, can regulate the AMPK-FoxO3a signalling pathway, leading to the activation of GABARAP transcription, we investigated whether metformin can regulate the membrane trafficking of $GABA_A$ receptors and thus have potential therapeutic value for anxiety, apart from its antidiabetic effects. To address this issue, we assessed the role of metformin in the membrane trafficking of $GABA_A$ receptors, by activation of the AMPK-FoxO3a-GABARAP signalling pathway, and in the consequent therapeutic effects on the hippocampus-dependent anxiety-like behaviours.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). All animal care and experimental procedures were in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology (HUST).

Male Sprague–Dawley rats (220–250 g) were obtained from the Animal Center of Tongji Medical College of HUST (Wuhan, Hubei, China). All the rats were housed in groups of three per cage (width 26.3 cm; depth 42.6 cm; height 20.2 cm) with clean sawdust bedding and acclimatized for 1 week under standard conditions [12:12h light cycle; a constant temperature (23 ± 1°C) and relative humidity (60 ± 5%)] with food and water available *ad libitum* (unless otherwise specified).

The rats were randomly assigned according to their body weights; all experiments were carried out in a blinded manner, compliant with Curtis *et al.*, (2018). Six hundred adult rats were used in this study, and 14 were lost during the experiments (post-operative death and incorrect cannula placement). The details of the numbers of rats used in *in vivo* experiments are shown in Supporting Information Table S1. The numbers (n) for each group used in *in vitro* study were shown in Supporting Information Table S2.

Primary hippocampal neuron cultures

P0-P1 Sprague–Dawley rat pups (n = 450) of either sex were obtained from the Animal Center of Tongji Medical College of HUST (Wuhan, Hubei, China). The rat pups were decapitated, the hippocampus was dissected from the brain and subsequently digested in PBS containing 0.125% trypsin (Amresco, OH, USA) at 37°C for 30 min. Tissues were mechanically triturated by repeated passages through a 5 mL pipette. Cell debris was removed by passing cells through a

nylon filter. The cell suspension was centrifuged at 300× *g* for 6 min at room temperature. After resuspension, dissociated hippocampal neurons were plated on poly-L-lysine-coated cover slips. Hippocampal neurons were kept in culture at 37°C and 5% CO_2/95% air in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with 5% FBS (Invitrogen) for 24 h and subsequently maintained in Neurobasal medium (Invitrogen) supplemented with 2% (v/v) B-27 (Invitrogen) and 2 mM GlutaMAX (Invitrogen). All experiments were performed on cultures from 10 to 14 days *in vitro* (DIV) and taken from at least three independent cultures obtained from different rats.

Drug treatment of neuronal cultures

Analyses of metformin concentrations in CA1 region were performed after rats were injected i.p. with the anxiolytic dosage of metformin (100 mg·kg^{-1}). Metformin (diluted in PBS, Sigma-Aldrich, St. Louis, MO, USA) was applied at a concentration of 10 μM *in vitro* experiment according to the result of MS/LC–MS analysis. A cell-permeable TAT-conjugated peptide was designed to disrupt GABARAP-$GABA_A$ receptor binding. The peptide contained the sequence RTGAWRHGRIHIRIAKMDGGGYGRKKRRQRRR. The scrambled control peptide contained the sequence HARHRGWHRIKIDIGRATGGGYGRKKRRQRRR (Marsden *et al.*, 2007). The peptides were purchased from GL Biochem (Shanghai, China, diluted in PBS) and applied at a concentration of 10 μM for 30 min before metformin treatment. The AMPK inhibitor, **Compound C** (Tocris, Bristol, UK) was applied at 20 μM for 30 min before treatment with metformin.

Drug treatment of rats in vivo

Metformin (50, 100, 200 mg·kg^{-1}) was dissolved in 0.9% NaCl, and **diazepam** (1 mg·kg^{-1}) was dissolved in DMSO and then injected i.p. 30 min before the behavioural test. The TAT-GABARAP inhibitory and scrambled peptides (as mentioned above) were dissolved in 0.9% NaCl and infused into the dorsal hippocampal CA1 region (30 pmol per side) bilaterally through the injection cannula connected to a 10 μL Hamilton syringe 30 min before treatment with metformin (100 mg·kg^{-1}, i.p.).

Gene silencing for cultured neurons

Hippocampal neuron cultures at DIV 8 were transfected with a negative control short hairpin RNA (shRNA) or shRNAs against **AMPKα$_2$** or FoxO3a for 72 h according to the manufacturer's specifications. Lentiviral vectors expressing the rat AMPKα_2 subunit and the FoxO3a shRNA were constructed by Genechem Co. Ltd. (Shanghai, China). The shRNA sequences targeting the rat AMPKα_2 sequence (GenBank NM_023991) was designed as follows: 5′-GCTGACTTCGG ACTCTCTA-3′. The shRNA sequences that target the rat FoxO3a sequence (GenBank NM_001106395) was designed as follows: 5′-GCCACAGCGATGTCATGAT-3′. The randomly chosen nonsense sequence: 5′-TTCTCCGAACGTGTCACGT-3′ was used as a negative control. The sequences of all of the constructs were confirmed by sequencing.

Surgery and microinjections of drugs

Cannula implantation into the CA1 region. Male Sprague–Dawley rats (220–250 g) were anaesthetized with sodium pentobarbital (i.p., 50 mg·kg^{-1}) and then mounted on a stereotaxic apparatus. The scalp was opened, the skull exposed and washed with 75% ethanol and 1% H_2O_2. Then a cannula made from 22 gauge stainless steel tubing (RWD Life Science, Shenzhen, China) was bilaterally implanted into the dorsal hippocampal CA1 region (−3.8 mm anterior posterior, ±3.0 mm medial lateral, −2.4 mm dorsal ventral from dura). A dust cap was used to cover the external end of the dummy cannula. The cannula was secured with dental cement. After the incisions were sutured, the rats were returned to their cages and allowed to recover for at least 7 days. All instruments were autoclaved, and the skin of the animals was sterilized with alcohol and iodine to prevent post-operative infection. After a recovery period of a week, the dummy cannula was removed, and an injection cannula protruding 1.0 mm beyond the tips of the guide cannula was inserted into the brain. The intracerebral infusion was performed through the injection cannula connected to a 10 μL Hamilton microsyringe. The TAT-GABARAP inhibitory and scrambled peptides (as mentioned above) were dissolved in 0.9% NaCl and infused into the dorsal hippocampal CA1 region (30 pmol per side) bilaterally 60 min before the behavioural test.

For intra-hippocampal microinjection of shRNA. Briefly, rats were anaesthetized and placed to the stereotactic apparatus, and then the skull was exposed as described above. Rats were randomly divided into three groups and microinjected with saline (n = 38), shRNA-control (n = 26), shRNA-FoxO3a (n = 20) or shRNA-AMPK (n = 16). A total volume of 4 μL lentivirus-shRNA were delivered bilaterally into the CA1 region at 0.5 μL·min^{-1} by a glass capillary with tip resistance of 5–10 MΩ connected to a syringe pump in the stereotaxic apparatus, followed by 10 min of rest to allow diffusion. Behavioural testing commenced 2 weeks followed virus injection, and saline and metformin (100 mg·kg^{-1}) were injected i.p. 30 min before the behavioural test.

Behavioural tests

Open field test (OFT). The open field apparatus was consisted of an opaque black acrylic sheet (1 m × 1 m × 0.3 m) and used to assess locomotor and anxiety behaviours. The ground area of the box was divided into the central area and the surrounding border zone. After pretreatment (30 min) with saline or metformin (100 mg·kg^{-1}, i.p.), the rat was randomly chosen and placed into the centre of the field and left to explore for 10 min. Behaviour in the open field arena was recorded using a CCD camera. Behavioural parameters were recorded and analysed by a behavioural tracking system (Anilab Software & Instruments, Ningbo, China). Basal exploration activity was measured by the total distance travelled (cm); anxiety-related behaviour was assessed by the duration and the travelled distance in the centre square. The surface of the open field arena was cleaned with 70% ethyl alcohol (EtOH) after each trial to remove permeated odours from previous animals. To select the anxiolytic dose of metformin, rats (n = 38) were

randomly divided into four groups: saline (n = 9), metformin 50 mg·kg^{-1} (n = 9), metformin 100 mg·kg^{-1} (n = 11) and metformin 200 mg·kg^{-1} (n = 9). Similarly, for diazepam, rats (n = 18) were randomly assigned to three groups: saline (n = 6), vehicle (n = 6) and diazepam 1 mg·kg^{-1} (n = 6). To test for tolerance to diazepam, rats (n = 18) were randomly assigned to the three groups described above.

Elevated plus maze test. The apparatus was constructed from a black acrylic sheet. Four arms (50 cm long and 10 cm wide) were connected and elevated to a height of 50 cm from the floor. Two arms were open (open arms), and the other two arms were enclosed within 40 cm walls. At the beginning of each trial, a rat, pretreated (30 min) with metformin (100 mg·kg^{-1} i.p.) or saline, was placed into the intersection of the four arms (centre area) facing an open arm. Behaviour in elevated plus maze was recorded for 5 min using a CCD camera. The behavioural parameters were recorded and analysed by a behavioural tracking system (Anilab Software & Instruments). Anxiety-related behaviour was deduced from the time spent in the open arms and the number of entries into the open arms. The surface of the elevated plus maze was cleaned with 70% EtOH after each trial in order to remove permeated odours from previous animals.

T-maze test. The rats were housed with restricted food, and their body weights were limited to 85% of their initial value. Before testing, rats were fed with food pellets in their home cage. The tests were carried out in a wooden T-maze, which was made up of a start stem (50 × 10 cm) and two goal arms (40 × 10 cm), surrounded by a 30 cm high wall. A guillotine door was mounted 20 cm from the stem. Guillotine doors were also placed at the entrance of each arm. A food dish was located at the end of each goal arm. The rats were allowed to freely explore for 10 min and eat food pellets at the maze for five successive days. Rats were trained to run from the start stem to one of the goal arm to get a pellet (six trials per day for 5 days). A testing trial contained a sample run (i.e. rats were forced to enter one of the goal arms and after consuming a pellet returned to the start stem) and a choice run. On every choice run, a correct choice was recorded when the rat entered the goal arm with pellet and consuming the pellet, and an error choice was recorded when rats did not enter the goal arm with the pellet. The goal arm with a pellet was not alternated until the rat did succeed. On the last day, the rats (n = 20, 10 rats per group) were assigned to metformin (100 mg·kg^{-1}, i.p.) or saline. After 30 min, the behavioural test was performed. A percentage or proportion correct of choice per animal can be calculated to assess spatial working memory.

Fear conditioning. Fear conditioning was assessed according to previous protocols (Wang *et al.*, 2011; Chen *et al.*, 2016) with some modification , using 10 rats in each group. Rats, pretreated (30 min) with saline or metformin (100 mg·kg^{-1}, i.p.) were allowed to adapt to their environment for 2 min and then presented with four pairings of a tone for 29 s as the conditioned stimulus (CS; 80 dB) that was matched with a foot shock as the unconditioned stimulus (US; 0.75 mA, 1 s). The inter-trial interval was 30 s. Rats were left in the conditioning chamber for 30 s after termination of the procedure and then returned to their home cage. To assess hippocampus-dependent contextual fear memory, 24 h after conditioning, rats were placed into the conditioning chamber and observed for 3 min. One hour later, the animals were assessed for amygdala dependent cued fear conditioning in a novel test chamber with a parameter consisting of four CS tones (30 s each with a 30 s intertrial interval).

EEG electrode placement and recording. Extradural EEG electrodes were surgically implanted under pentobarbital sodium anaesthesia as described (Chemali *et al.*, 2012). Briefly, rats were anaesthetized and placed in a stereotactic frame. The skull was exposed and four small stainless steel electrodes (diameter 0.6 mm) were positioned with their tips just touching the dura mater at stereotactic coordinates: (electrode 1) A (anterior) 6 (mm) L (lateral) 3 (mm), (electrode 2) A6L-3, (electrode 3) A0L0, (electrode 4) A10L2 relative to the lambda. The electrodes, wired to a small connector, were fixed with dental cement leaving access only to the upper part of the connector, and the skin was closed. Postoperative analgesia was provided, and animals were allowed to rest for a minimum of 7 days to fully recover. The EEG signal was recorded using a QP511 Quad AC Amplifier System (Grass Instruments, West Warwick, RI, USA) and a USB-6009 14-bit data acquisition board (National Instruments, Austin, TX, USA). The sampling rate was 500 Hz, and data were bandpass filtered between 0.3 and 50 Hz. A line filter was used to eliminate 60 Hz noise. The record lasted at least 30 min as baseline for subsequent experiments. Rats (n = 10, five rats per group) were pretreated (30 min) with saline or metformin (100 mg·kg^{-1}, i.p.). The EEG was recorded for 30 min after injection.

Blood glucose measurement. The rats were fasted for 12 h, and the blood glucose levels were measured at 0.5, 1, 2 and 4 h after treatment with saline or metformin (100 mg·kg^{-1}, i.p.). Blood was obtained *via* tail incision and measured using an Advantage Accu-Chek glucometer (Boehringer Mannheim Corp., Indianapolis, CA, USA). Rats (n = 62) were randomly divided into saline and metformin groups respectively.

LC–MS/MS analysis for quantification of metformin in rats

Rats (n = 42) were randomly assigned to seven groups (six rats per group), according to the sampling time and a single dose of metformin (100 mg·kg^{-1}) was injected i.p.. Plasma, CSF and hippocampus tissue samples were obtained at 0.25, 0.5, 0.75, 1, 2, 6 and 12 h after metformin injection. Briefly, rats were anaesthetized and fixed in a stereotaxic frame after each point of metformin administration; the needle was punctured into the cisterna magna (depth: 7 mm) for CSF collection. The blunt end of the needle was connected to the plastic collection syringe by PE-50 tubing. The samples of transparent CSF (60–100 μL per rat) were stored at −80°C. Blood samples (2 mL) and hippocampus tissue samples were taken after decapitation and after CSF collection. The blood sample was collected into plastic tubes moistened with heparin and centrifuged (2500× g, 4°C, 15 min) to obtain plasma. Transparent plasma was transferred into new tubes and

immediately frozen at −80°C. Hippocampus tissue samples were separated from brain, weighed, transferred into plastic tubes and stored at −80°C. Plasma was diluted 10- or 100-fold with deionized water, while CSF was diluted fivefold with water. For brain tissue samples, approximately 100 mg of brain tissue was homogenized in three volumes of water;To 100 μL of each resultant matrix of interest was added 10 μL internal standard (IS, 40 $\mu g \cdot mL^{-1}$ huperzine A solution in methanol), followed by 300 μL of acetonitrile for sample clean-up. The mixture was vortexed for 5 min and centrifuged at 22 136× *g* for 10 min. Supernatant (10 μL) was applied for LC–MS/MS analysis on an Applied Biosystem 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA) coupled with a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan). Metformin was eluted through a Waters UPLC C18 column (1.7 μm, 2.1 mm × 100 mm; Waters Ltd. Corp., USA) using isocratic elution at a flow rate of 0.3 $mL \cdot min^{-1}$, with a mixture of acetonitrile and 10 mM ammonium formate (60:40, v/v) as the mobile phase. The column and autosampler temperatures were set at 40°C and 4°C respectively. The mass spectrometer equipped with an electrospray ionization (ESI) source was operated in positive ion multiple reaction monitoring mode through monitoring the ion transitions of m/z 130.1 → 60.0 and m/z 136.1 → 60.0 for metformin and d_6-metformin (internal standard) respectively. The ESI source settings for the ionization of metformin were as follows: ion spray voltages: 5000 V; source temperature (TEM): 550°C; curtain gas (CUR): 30 psi; nebulizer gas: 40 psi; auxiliary gas: 30 psi. The assay was fully validated with a dynamic range of 1–2500 $ng \cdot mL^{-1}$ ($r > 0.999$). The concentration of the d_6-metformin was 250 $ng \cdot mL^{-1}$. Analyst 1.6.1 software (Applied Biosystem/MDS Sciex) was utilized for the instrument control, data acquisition and processing. Pharmacokinetic parameters were calculated by non-compartmental analyses using DAS 2.1.1 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Electrophysiological recording

In cultured neurons. GABAergic currents were recorded from cultured hippocampal neurons using whole-cell patch-clamp methods as previously described (Rannals and Kapur, 2011). The membrane properties and synaptic currents of DIV 12 hippocampal neurons from these cultures have been previously characterized. Thick-walled borosilicate patch electrodes (World Precision Instruments, Sarasota, USA) were pulled to a low final resistance of 3–5 MΩ using a dual-stage glass micropipette puller PC-10 (Narishige, Tokyo, Japan). Pyramidal neurons were visually identified by their morphology with an inverted microscope. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded at room temperature with an EPC10 Amplifier (HEKA Elektronik, Lambrecht, Germany) using patch electrodes filled with a recording solution containing (in mM): 153.3 CsCl, 1.0 $MgCl_2$, 5.0 EGTA and 10.0 HEPES, with a pH of 7.2 and an osmolarity of 290–300 mOsm. MgATP (4 mM) was added to the intracellular solution before recording. The external solution for the recordings contained (in mM): 146.0 NaCl, 2.5 KCl, 3.0 $MgCl_2$, 2.0 $CaCl_2$, 10.0 glucose and 10.0 HEPES, with a pH of 7.40 and an osmolarity of 315–330 mOsm. Internal and external recording solutions contained equimolar concentrations of chloride ions, and at a clamped membrane potential of 0 mV, no $GABA_A$ receptor-mediated mIPSCs were present. For recording mIPSCs, glutamate receptor-mediated synaptic currents were blocked by 50 μM D-2-amino-5-phosphonovalerate (D-APV) and 20 μM **6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)** in the external solution, and action potentials were blocked by adding 1 μM **tetrodotoxin (TTX)** in the external solution. Neurons were voltage-clamped to −60 mV. The $GABA_A$ receptor antagonist **bicuculline** (10 μM) was bath applied, and the elimination of observed currents demonstrated that GABAergic currents were recorded. mIPSCs were collected continuously for 6 min before and 30 min after metformin (10 μM) application. mIPSCs were analysed using the Mini Analysis Program (Synaptosoft, Decatur, USA). Data points were obtained by binning mIPSCs data in 2 min intervals and normalizing to the mean of the baseline amplitude/frequency time point.

In slices. Hippocampal slices (300–350 μm thick) from 7- to 12-week-old rats were used for mIPSC recordings. Rats were anaesthetized with pentobarbital sodium (60 $mg \cdot kg^{-1}$, i.p.) and perfused with ice-cold cutting solution (in mM: 209 sucrose, 22 glucose, 1.25 NaH_2PO_4, 3.1 sodium pyruvate, 12 sodium L-ascorbate, 4.9 $MgSO_4 \cdot 7H_2O$ and 26 $NaHCO_3$; oxygenated with 95% O_2 and 5% CO_2, pH 7.2–7.4). Coronal hippocampal slices were cut by a vibrating blade microtome (VT1000S, Leica, Wetzlar, Germany) in ice-cold cutting solution. Slices were incubated in aCSF (in mM: 128 NaCl, 2 $MgCl_2$, 3 KCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 10 D-glucose and 2 $CaCl_2$; oxygenated with 95% O_2 and 5% CO_2, pH 7.2–7.4, 295–305 mOsm) for 1 h at 28°C and then returned to the room temperature for recording. Patch pipettes (3–6 MΩ) for whole-cell voltage-clamp recordings were filled with internal solution (in mM: 153.3 CsCl, 1 $MgCl_2 \cdot 6H_2O$, 5 EGTA, 10 HEPES and 4 MgATP; pH 7.2, 285 mOsm). Whole-cell recordings were obtained from the CA1 region pyramidal cells. mIPSCs were recorded in voltage-clamp mode (Vh = −70 mV). All data were acquired from a Multiclamp 700B amplifier and pCLAMP 10 software (Axon Instruments, Molecular Devices, USA) for recording mIPSCs. Records were low pass-filtered at 2–20 kHz and digitized at 5–50 kHz. The mIPSCs were recorded in the presence of TTX (10 μM) and CNQX; (10 μM) and D-APV (50 μM).

Immunofluorescence experiments for cultured neurons

Assay for surface level of $GABA_A$ receptors. Hippocampal neurons were treated with metformin (10 μM) for 60 min at 37°C. Cells on the coverslips were washed with PBS (pH 7.2) then fixed *in vitro* with 4% paraformaldehyde in PBS for 20 min. The coverslips were washed three times (10 min) with PBS, blocked with 3% normal donkey serum for 30 min at room temperature and then incubated with anti-**$GABA_A$ receptor γ_2 subunit** antibody (1:200, sc-131935, Santa Cruz, CA, USA) overnight at 4°C. Coverslips were washed three times with PBS and incubated with a donkey anti-goat DyLight 594 fluorochrome-conjugated secondary antibody (1:50, Pierce, Rockford, IL, USA) for 60 min while

shaking at room temperature in the dark. The coverslips were washed three times with PBS and incubated for 2 min with Hoechst 33258 nucleic acid stain (1:5000 diluted in PBS, Invitrogen). Finally, the coverslips were mounted on slide glasses. All primary and secondary antibodies were diluted in PBS containing 1% normal donkey serum.

Assay for FoxO3a nuclear translation. Hippocampal neurons were treated with Compound C (20 μM) for 30 min before metformin (10 μM) treatment for 15 min at 37°C. The cells were fixed with 4% paraformaldehyde for 20 min and then washed three times (10 min) with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature, blocked with 3% normal goat serum and 0.3% Triton X-100 in PBS for 30 min and incubated overnight at 4°C with a rabbit anti-FoxO3a primary antibody (1:200, Cell Signalling Technologies, Beverly, MA, USA). The coverslips were washed three times (10 min) with PBS, incubated with a goat anti-rabbit DyLight FITC-conjugated secondary antibody (1:50, Pierce) for 60 min while shaking at room temperature in the dark. The coverslips were washed with PBS and incubated for 2 min with Hoechst 33258 nucleic acid stain (1:5000 diluted in PBS, Invitrogen). Finally, the coverslips were mounted on slide glasses. All primary and secondary antibodies were diluted in PBS containing 1% normal goat serum and 0.3% Triton X-100. PBS was used as the controls for antibody. The images were collected using confocal laser scanning microscopy (LSM 710, Carl Zeiss, Germany). Image-Pro Plus (Media Cybernetics) was used to quantify the integrated OD.

Immunohistochemistry processing

After treatment with metformin (100 mg·kg^{-1}, i.p.) or saline for 30 min, rats were anaesthetized by sodium pentobarbital (50 mg·kg^{-1}, i.p.) and perfused transcardially with ice-cold PBS, followed by ice-cold PBS containing 4% paraformaldehyde fixative solution (Fluka, Sigma-Aldrich). The rats were then decapitated; the brains were rapidly removed and fixed in a solution containing of 4% paraformaldehyde for 48 h. All tissues were cryoprotected in 25% sucrose-PBS for 48 h and cut on a cryostat-microtome (Leica CM1900, Germany) in 25–30 μm coronal sections. Slices were collected and stored in six-well trays (Corning Inc., NY, USA), free-floating in PBS supplemented with 0.12 mM sodium azide. The plates were stored at 4°C until immunohistochemical processing.

The sections were blocked with 3% BSA (Sigma-Aldrich) for 30 min at room temperature, washed with PBS three times (10 min) and incubated with a goat anti- $GABA_A$ receptor γ_2 subunit antibody (1:200, Santa Cruz) overnight at 4°C. Sections were then washed with PBS three times (10 min) and incubated overnight at 4°C with a second primary antibody, a mouse monoclonal antibody against gephyrin (1:200, BD Biosciences, Franklin Lakes, NJ, USA). Sections were again washed three times (10 min) with PBS and incubated with the daylight 594 fluorochrome-conjugated donkey anti-goat (1:50, Pierce) and FITC fluorochrome-conjugated donkey anti-mouse IgG (4 μg·mL^{-1}, Invitrogen) for 60 min while shaking at room temperature in the dark. Sections were then washed three times (10 min) with PBS. Subsequently, sections were counterstained with Hoechst 33258 (1:5000, Invitrogen) to identify nuclei for 2 min at room temperature and washed three times with PBS. All primary and secondary antibodies were diluted in PBS containing 1% normal donkey serum. Fluorescence images were acquired with a LSM710 Zeiss microscope for double staining experiments.

Chromatin immunoprecipitation assay

FoxO3a transcription factors share the consensus DNA-binding sequences TT(G/A)TTTTC and (C/A)(A/C)AAA(C/T)AA in the promoters of target genes (Furuyama *et al.*, 2000; Obsil and Obsilova, 2011). To determine whether FoxO3a binds directly to the promoter of the GABARAP gene or activates its transcription by an indirect mechanism, the chromatin immunoprecipitation (ChIP) was used to test whether FoxO3a was associated with the putative FoxO-binding sites in the proximal promoter regions of GABARAP. The promoter region of the GABARAP gene contains three putative FoxO3a binding sites: (i) CTTTGTTTTGTGT at −491/−498, (ii) TTTTGTTTGCAACA at −655/−662 and (iii) ATGGCAAACAGCTG at −852/−857.

Hippocampal neurons were treated with or without metformin (10 μM) for 30 min before analysis using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Cellular chromatin was cross-linked by 4% formaldehyde and then sheared by sonication. The soluble chromatin was immunoprecipitated with an anti-FoxO3a monoclonal antibody (Cell Signalling). After de-crosslinking, the immunoprecipitates were subjected to PCR. The relative levels of DNA were normalized to the input DNA and expressed as a percentage of the nontreated control. Primers for the GABARAP promoter were designed with the Primer premier 5.0 software and the NCBI BLAST program. The sequences used were the following: (i) forward: 5′-CATAGAAGGCT GAAGAGGAA-3′ and reverse: 5′-GGAGGTGCCAGGAAAG AG-3′; (ii) forward: 5′-TTGACTGGGAAGTTGACG-3′ and reverse: 5′-ATACCATAAGACGGAGAAGAA-3′; (iii) forward: 5′-TCCGCCTGAGTCTTGAGC-3′ and reverse: 5′-GTGGCA GTCGTGAGATTTAGTT-3′.

Surface receptor cross-linking assays

Surface expression of $GABA_A$ receptor γ_2 subunit was assayed using the membrane-impermeable cross-linking reagent bis (sulfosuccinimidyl) suberate (BS^3) as described previously (Boudreau and Wolf, 2005; Lu *et al.*, 2014).

For neurons. Briefly, after pretreatment (30 min) with metformin (10 μM), hippocampal neurons were immediately washed twice with 1× PBS. BS^3 (Pierce) was added at a concentration of 2 mM and incubated with the cells for 30 min at 4°C. The reaction was terminated by quenching with 200 mM glycine (15 min, 4°C). The samples were then washed three times (10 min) with PBS, collected in Eppendorf tubes containing ice-cold lysis buffer and homogenized to obtain the total protein homogenate and analysed directly by 8% SDS-polyacrylamide gel electrophoresis.

For CA1 tissues. After pretreatment (30 min) with saline or metformin (100 mg·kg^{-1} i.p.), brains were removed under anaesthetic and were cut into coronal sections (400 μm). The CA1 region was dissected and added to Eppendorf tubes containing ice-cold artificial CSF (ACSF). BS^3 (Pierce) was

added at a concentration of 2 mM, and the tubes were incubated for 30 min at 4°C. The reaction was terminated by quenching with 200 mM glycine (15 min, 4°C). The tissue was then washed four times (10 min) with ice-cold ACSF and pelleted by brief centrifugation at 4°C (5000 g, 5 min), resuspended in ice-cold lysis buffer, homogenized to obtain total protein homogenate and analysed directly by 8% SDS-polyacrylamide gel electrophoresis.

Coimmunoprecipitation

After drug treatments, hippocampal neurons (or CA1 tissues) were briefly washed with ice-cold PBS (or ACSF). Protein extracts were prepared by RIPA buffer (50 mM Tris–HCl, pH = 8, with 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) (P0013, Beyotime, Shanghai, China). Protein extracts (500–1000 μg) were then incubated with 2 μg of antibody to $GABA_A$ receptor γ_2 subunit or 2 μg of IgG overnight at 4°C with constant shaking. The antibody-bound complexes were incubated with Protein A/G agarose (sc-2003, Santa Cruz) for 2 h at 4°C. The protein-bound beads were washed in RIPA buffer three times (10 min) and pelleted by centrifugation for 3 min at 885× *g*. The beads were resuspended in 2× SDS sample buffer, and the immune complexes were eluted by boiling at 95°C for 5 min. Total and immunoprecipitated proteins were separated by electrophoresis on 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. The blots were probed with antibodies to GABARAP (Novus, Cambridge, MA, USA) diluted to 1:2000.

Western blot analysis. Western blot was performed according to a protocol used earlier (Li *et al*., 2018). Briefly, the cell and tissue extracts were prepared in RIPA buffer containing PMPS and protease inhibitors (v/v/v: 98:1:1) on ice for 30 min. Cell lysates were centrifuged at 12 000× *g* for 20 min at 4°C and assayed protein content with a BCA protein assay kit (Pierce); 30 μg of total protein were separated by 8–15% SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% BSA, incubated with antibody (diluted in 1% BSA) overnight at 4°C, incubated with secondary antibody (diluted in 1% BSA) for 1 h at room temperature after washed and analysed by Western blot using the ECL method (Pierce). We used primary antibodies against the following: AMPK (2532, 1:1000, Cell Signalling), p-AMPK (Thr172, 2535, 1:1000, Cell Signalling) and FoxO3a (2497, 1:1000, Cell Signalling); GABARAP (NBP1–95588, 1:1000, Novus) and $GABA_AR$ γ_2 (NB300–190, 1:1000, Novus); **$GABA_A$ receptor α1** (ab33299, 1:1000, Abcam, MA, USA); **$GABA_A$ receptor α_2** (ab72445, 1:1000, Abcam); **$GABA_A$ receptor β2** (ab8340, 1:1000, Abcam); **$GABA_{B2}$** (ab52248, 1:500, Abcam); **GluA1** (ab31232, 1:1000, Abcam); **GluA2** (ab20673, 1:500, Abcam); anti-**GluN2B** (ab65783, 1:1000, Abcam); anti-**mGluR5** (ab76316, 1:5000, Abcam); GAPDH (sc-365 062, 1:2000, Santa Cruz). Protein levels were quantified by using the image analysis software ImageJ (Version 1.46, NIH, Maryland, USA).

Data and statistical analysis

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2018). The data were analysed by an investigator blinded to the experimental conditions. Each *in vitro* experiment was conducted independently at least three times, and more than five animals per group were needed for *in vivo* experiment. All data were expressed as the means ± SEM. The statistical analyses were performed using SPSS 13.0 software. All data obtained from our experiments conformed to normal distribution as determined by Kolmogorov–Smirnov test. Potential differences between the mean values were evaluated using one-way or two-way ANOVA and followed by Bonferonni *post hoc* test. Independent sample *t*-tests were used to compare differences between any given two groups throughout the study, unless otherwise specified. $P < 0.05$ was considered to be statistically significant.

Materials

D-APV, CNQX, bicuculline, TTX and metformin were purchased from Sigma-Aldrich (St. Louis, USA). Diazepam was obtained from JiChuan pharmacology (Jiangsu, China).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al*., 2017a,b,c).

Results

Metformin rapidly ameliorates the anxiety-like behaviour in rats

We administered metformin (50, 100, and 200 $mg{\cdot}kg^{-1}$, i.p.) for 30 min and found that a dose of 100 $mg{\cdot}kg^{-1}$ displayed a robust anxiolytic effect as defined by increased duration in the centre square in the open field test (OFT, Figure 1A,B) when compared with saline-treated rats but without affecting locomotor activity (Figure 1C). These effects of metformin on behaviours were comparable to that of diazepam, which is a typical anxiolytic drug and was used as a positive control (Figure 1D–F). Furthermore, in the elevated plus maze (EPM) test, metformin (100 $mg{\cdot}kg^{-1}$, i.p. 30 min) increased the percentage of time spent in the open arms (Figure 1G,H) and total distance travelled (Figure 1G,I). However, when rats were treated with 200 $mg{\cdot}kg^{-1}$ of metformin for 30 min, they displayed lower centre duration and locomotor activity, indicating that metformin at this dosage might induce sedative effects in rats (Figure 1B,C). Taken together, these results suggest that metformin has a therapeutic potential for the rapid relief of anxiety disorders.

Next, we wondered whether metformin, like diazepam, could also easily induce tolerance during chronic treatment. In the OFT and EPM test, metformin (100 $mg{\cdot}kg^{-1}$, i.p., 30 min) significantly increased the time spent in the centre square (Figure 1J) and duration (Figure 1K) in open arms on day 1, and this effect was fully retained and even increased after 14 days of repeated treatment, whereas tolerance was observed in diazepam-treated rats after 14-days treatment (Supporting Information Figure S1). Moreover, 100 $mg{\cdot}kg^{-1}$ metformin had no effect on locomotor

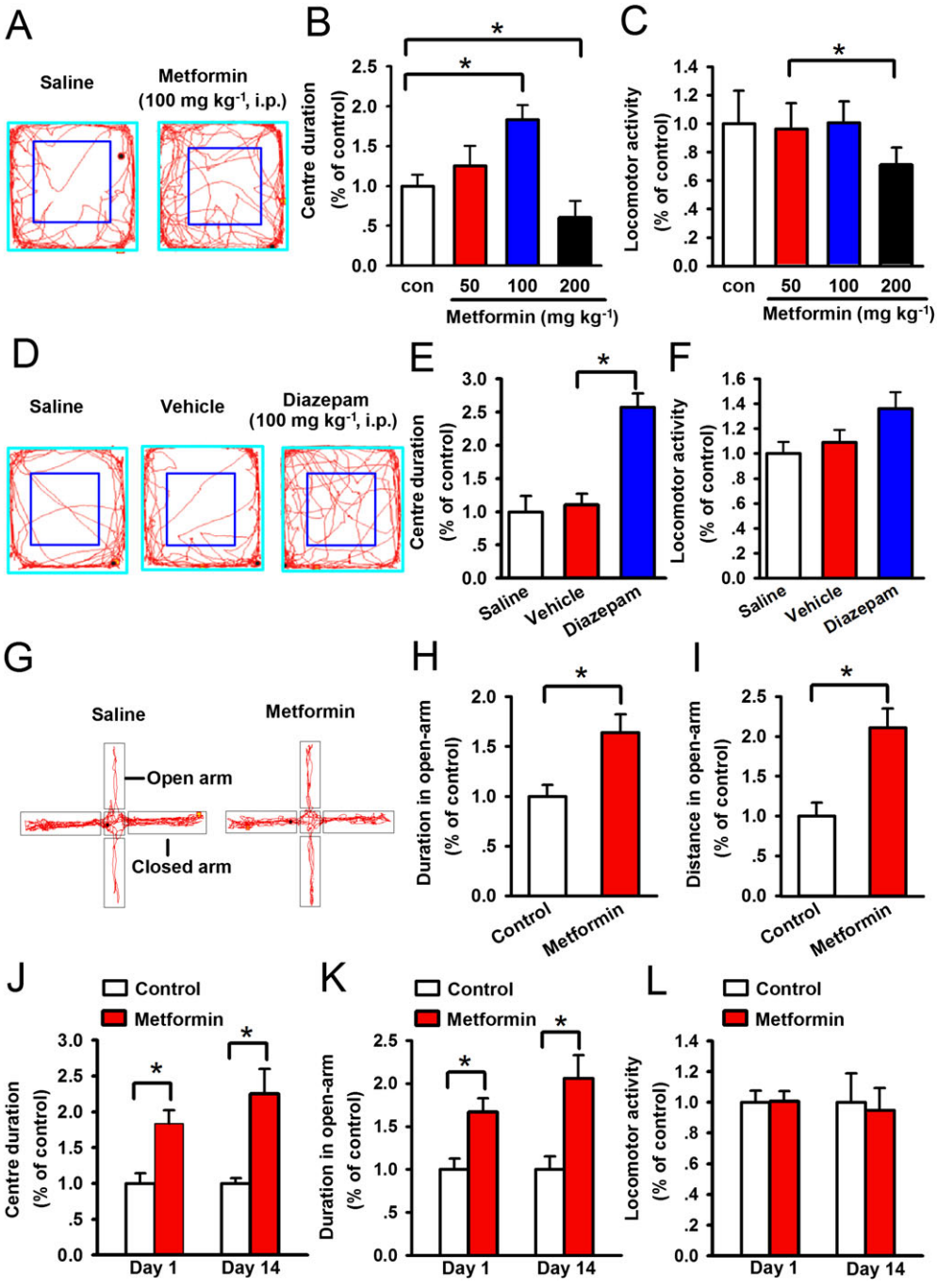

Figure 1

Metformin produces anxiolytic-like effects in rats. (A–C) During the OFT, metformin-treated (100 mg·kg^{-1}, i.p.) rats showed a significant increase in centre duration (B) but unchanged locomotor activity (C) (saline: $n = 9$; metformin 50 mg·kg^{-1}: $n = 9$; 100 mg·kg^{-1}: $n = 11$; 200 mg·kg^{-1}: $n = 9$). (D–F) Diazepam treatment (1 mg·kg^{-1}, i.p.) of rats also enhanced the duration (E) in the centre square but not the locomotor activity (F) ($n = 6$ per group). (G–I) During the EPM, the metformin-treated (100 mg kg^{-1}, i.p.) rats displayed a significantly greater percentage of duration in the open arm (H) and longer distance (I) ($n = 12$ per group). (J–K) Both single and chronic (14 days) metformin treatment displayed anxiolytic-like effect on OFT (J, day 1: $n = 11$; day 14: $n = 10$ per group) and EPM (K, day 1: $n = 8$; day 14: $n = 7$ per group). (L) No significant differences among groups were found in total travelled distance in OFT ($n = 10$ per group). Data are expressed as means ± SEM. One-way ANOVA followed by Bonferroni *post hoc* test,*$P < 0.05$, significantly different from control.

activity (Figure 1L) and blood glucose level (Supporting Information Figure S2A,B) at days 1 and 14 and did not affect the EEG and working memory of rats (Supporting Information Figure S2C,D). We also found that metformin (100 mg·kg^{-1}, i.p., 30 min) promoted contextual fear memory but not cue fear memory (Supporting Information

Figure S2E). These results suggest that metformin is not prone to induce tolerance, and its therapeutic effect is not mediated by a general inhibitory effect.

Metformin increases the surface expression of $GABA_A$ receptors and inhibitory synaptic transmission in cultured rat hippocampal neurons

To explore the mechanism(s) underlying the anxiolytic effects of metformin, we sought to determine whether metformin could cross the blood–brain barrier. Figure 2A shows the time-concentration curve of metformin in the hippocampus after a single i.p. injection of 100 $mg \cdot kg^{-1}$ metformin. It reached a peak concentration (7.4 ± 3.3 μM) in the hippocampus at 15 min after administration, demonstrating a rapid distribution of metformin throughout the hippocampus from the systemic circulation, indicating that it can readily penetrate the blood–brain barrier. The pharmacokinetic parameters in the hippocampus, plasma and CSF are shown in Supporting Information Figure S3 and Table S3. In order to examine the mechanism of the anxiolytic effect of metformin, a concentration of 10 μM (near the peak concentration in the hippocampus) was applied in the *in vitro* experiments.

Increasing evidence shows that redistribution of $GABA_A$ receptors is involved in some forms of inhibitory synaptic plasticity. Over 90% of $GABA_A$ receptors include the γ_2 subunit, which is the most abundant $GABA_A$ receptor subunit in the CNS (Smith and Rudolph, 2012). To examine whether metformin had an effect on $GABA_A$ receptor redistribution, immunofluorescence analysis was performed in cultured hippocampal neurons under non-permeabilizing conditions. Treatment with metformin (10 μM) for 30 min induced a significant increase in the numbers of surface puncta of the $GABA_A$ receptor γ_2 subunit (Figure 2B). Furthermore, we demonstrated that the bis (sulfosuccinimidyl) suberate (BS^3) cross-linking assay was suitable for $GABA_A$ receptors in cultured hippocampal neurons (Supporting Information Figure S4A). Incubation with metformin (10 μM, 30 min) increased the surface expression of the $GABA_A$ receptor γ_2 subunit in hippocampal neurons (Figure 2C). However, the total amount of $GABA_A$ receptor γ_2 subunit and the surface level of excitatory glutamate receptors remained unchanged (Figure 2D and Supporting Information Figure S4B). These results were also supported by electrophysiological experiment. The patch-clamp recording showed that bath application of metformin (10 μM) for 30 min induced a significant increase in the average amplitude (Figure 2F,H) and frequency (Figure 2G,I) of mIPSC (Figure 2E) in cultured hippocampal neurons. Taken together, these findings indicate that metformin increased the inhibitory neurotransmission in cultured hippocampal neurons.

Metformin-induced membrane insertion of $GABA_A$ receptors is mediated by increasing GABARAP expression and its interaction with $GABA_A$ receptor γ_2 subunit

As metformin induced a redistribution of $GABA_A$ receptors, we next explored the mechanism underlying the increase in the surface expression of $GABA_A$ receptors induced by metformin. Previous studies have shown that GABARAP plays a central role in the trafficking of $GABA_A$ receptors in cultured neurons (Leil *et al.*, 2004; Marsden *et al.*, 2007; Luscher *et al.*, 2011a). Thus, we speculated that GABARAP might be involved in the metformin-induced increase in surface expression of $GABA_A$ receptors . As shown in Figure 3A,B, metformin (10 μM, 30 min) increased the expression of GABARAP without affecting the total amount of $GABA_A$ receptor γ_2 subunit and increased the interaction between the $GABA_A$ receptor γ_2 subunit and GABARAP (Figure 3C). When the neurons were pretreated with the cell-permeable TAT-conjugated peptide for 30 min to disrupt the interaction between GABARAP and $GABA_A$ receptors , the surface levels of $GABA_A$ receptors induced by metformin were markedly decreased (Figure 3D,E), without affecting the basal expression of surface $GABA_A$ receptors (Supporting Information Figure S6E).

FoxO3a is involved in the metformin-induced increase in the surface expression of $GABA_A$ receptors

GABARAP is initially identified as a molecular chaperone for $GABA_A$ receptors (Wang *et al.*, 1999). In addition, GABARAP also belongs to the Atg8 (autophagy-related protein 8) family, which includes LC3B, GABARAPL1 and GATE-16, and it is critical for autophagosome biogenesis and maturation (Chen and Olsen, 2007; Weidberg *et al.*, 2010). Increasing evidence indicates that FoxO3a is a major player in the transcriptional regulation of autophagy-related genes, including GABARAP (Mammucari *et al.*, 2007; Chiacchiera and Simone, 2010). Therefore, we asked whether FoxO3a regulated GABARAP transcription in neurons as well. Consistent with previous studies, the expression of FoxO3a protein was increased after metformin (10 μM, 30 min) treatment (Figure 3A,B). The ChIP assay was used to test whether FoxO3a was associated with the putative FoxO-binding sites in the proximal promoter regions of GABARAP (Furuyama *et al.*, 2000; Obsil and Obsilova, 2011). The results from a ChIP assay with the FoxO3a antibody showed that FoxO3a strongly bound to site 3 (Figure 4A). Importantly, the binding of FoxO3a to the GABARAP promoter was significantly increased by metformin treatment (Figure 4B), suggesting that FoxO3a may mediate metformin-induced GABARAP transcription by a direct mechanism. Further, short hairpin RNA FoxO3a (shRNA-FoxO3a) was used to silence FoxO3a expression (Figure 4C). We found that shRNA-FoxO3a prevented metformin-induced up-regulation of GABARAP expression as well as the surface level of $GABA_A$ receptors (Figure 4D,E). Therefore, our results strongly suggest that FoxO3a plays a critical role in metformin-induced GABARAP expression and insertion of $GABA_A$ receptors into the neuronal cell membrane.

AMPK is required for metformin-induced FoxO3a activation and membrane insertion of $GABA_A$ receptors

Metformin has been demonstrated to activate the AMPK signalling pathway, which is involved in FoxO3a activation (Greer *et al.*, 2007; Chiacchiera and Simone, 2010; Sato *et al.*, 2012). We found that metformin increased the phosphorylation of AMPK in cultured hippocampal neurons (Figure 3A,B). The immunofluorescence results showed that the AMPK inhibitor Compound C (20 μM, preincubated for

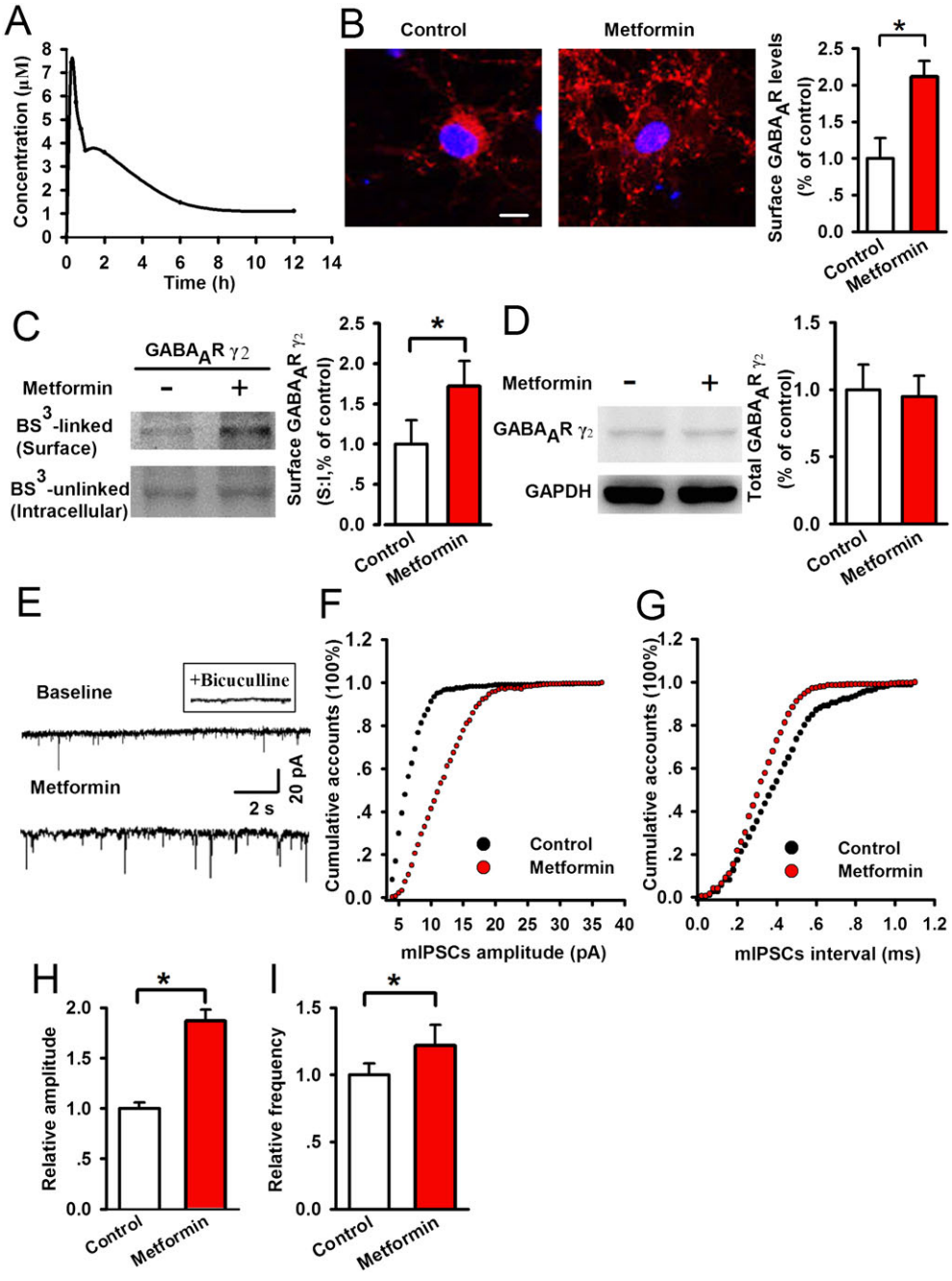

Figure 2

Metformin increases the surface expression of $GABA_A$ receptors and inhibitory synaptic transmission in cultured hippocampal neurons from rats. (A) The mean concentration of metformin in the hippocampus of rats after a single intraperitoneal injection (100 mg·kg^{-1}) (n = 6 per group). (B) Immunofluorescence analysis showing that metformin (10 μM) significantly increased the surface expression of $GABA_A$ receptor γ_2 subunit (red) in cultured hippocampus neuron. Hoechst 33258 (blue). Scale bars: 20 μm. n = 10 per group. (C–D) Metformin (10 μM, 30 min) significantly increased the surface expression of $GABA_A$ receptor γ_2 subunit of hippocampal neurons (C) but not the total expression of the protein (D) (n = 5 per group). (E–G) Representative traces of mIPSCs (E), cumulative plots of mIPSCs amplitude (F) and inter-event interval (G) in cultured hippocampal neurons during the application of 10 μM metformin. (H–I) Bar plot summary showing metformin significantly increased the amplitude (H) and frequency (I) of mIPSCs (n = 10 per group). Data are expressed as means ± SEM. *$P < 0.05$, significantly different from control.

30 min) significantly blocked the metformin-induced nuclear translocation of FoxO3a (Figure 5A,B). Pretreatment with the AMPK inhibitor Compound C blocked metformin-induced the increase in GABARAP expression (Figure 5C) and the surface expression of $GABA_A$ receptors (Figure 5D). We also found that the shRNA-AMPKα_2 reduced AMPKα_2 protein expression (Figure 5E) and inhibited metformin-induced increase in the expression of FoxO3a (Figure 5F), GABARAP (Figure 5G) and the surface level of $GABA_A$ receptors (Figure 5H). Taken together, these results suggest that the

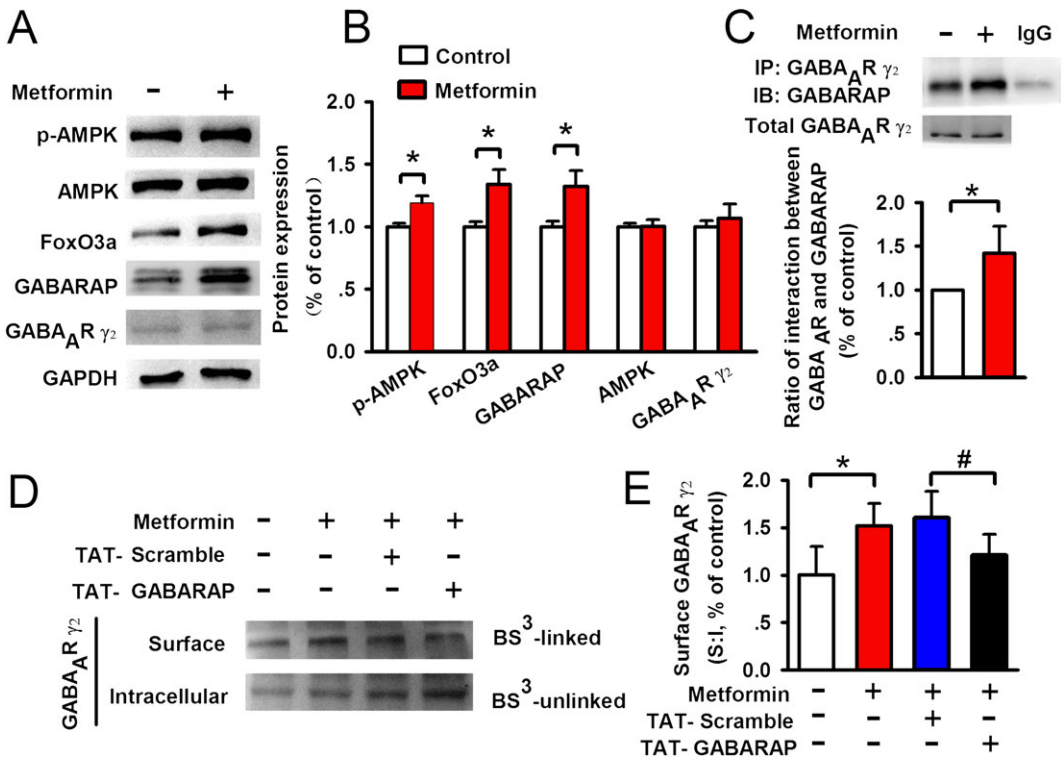

Figure 3

Metformin-induced increase in surface expression of $GABA_A$ receptors is mediated by elevated expression of GABARAP and its interaction with $GABA_A$ receptor γ_2 subunits in cultured hippocampal neurons. (A–B) Western blot analysis showed that metformin (10 μM, 30 min) significantly increased the protein expression of p-AMPK, FoxO3a, GABARAP. (C) Co-IP showed that metformin enhanced the binding of $GABA_A$ receptor γ_2 to the GABARAP, without affecting the total $GABA_A$ receptor γ_2 subunits. (D–E) The GABARAP inhibitory peptide (TAT-GABARAP) pretreatment inhibited the surface expression of $GABA_A$ receptor γ_2 subunits induced by metformin. Data are expressed as the means ± SEM. $n = 6$ per group, one-way ANOVA with Bonferonni *post hoc* test. $^*P < 0.05$, significantly different from control; $^{\#}P < 0.05$, significantly different from TAT-scramble peptide.

activation of AMPK by metformin increased FoxO3a transcription activity and facilitated the translocation of $GABA_A$ receptors to the neuronal cell surface.

The membrane insertion of $GABA_A$ receptors by the AMPK-FoxO3a-GABARAP pathway contributes to metformin-induced anxiolytic-like behaviours

Our *in vitro* study determined the mechanism by which metformin increased the surface expression of $GABA_A$ receptors in cultured hippocampal neurons. Next, we examined whether the mechanism of the membrane insertion of $GABA_A$ receptors was also involved in metformin-induced anxiolytic-like behaviours. As shown in Figure 6A, metformin (100 mg·kg^{-1}, i.p., 30 min) led to a significant increase in the density of the $GABA_A$ receptor γ_2 subunit cluster in the dorsal hippocampal CA1 region when compared with saline-treated rats (Figure 6B). Using tagged gephyrin as a marker of inhibitory synapses, we found that the puncta sizes of gephyrin were larger in metformin-treated rats than that in saline-treated rats (Figure 6C). Also, the colocalization between the $GABA_A$ receptor γ_2 subunit and gephyrin was increased in metformin-treated rats (Figure 6D). Furthermore, the patch-clamp recordings showed that metformin increased the amplitude and frequency of mIPSCs in the dorsal hippocampal CA1 regions (Figure 6E–G and Supporting Information Figure S6A,B). In addition, the changes in surface expression of $GABA_A$ receptors induced by metformin *in vivo* were investigated by BS^3 cross-linking assay. When compared with saline treatment, metformin (100 mg·kg^{-1}, i.p., 30 min) induced an increase in protein abundance of the $GABA_A$ receptor γ_2 subunit in the surface pool of dorsal hippocampal CA1 region (Figure 6H,I). However, the total amount of the $GABA_A$ receptor γ_2 subunit remained unchanged (Figure 6J). Metformin also improved membrane trafficking of the $GABA_A$ receptor α_1 and α_2 subunits but without effects on $GABA_B$ receptor subunit 2 ($GABA_{B2}$) and glutamate receptor subunits (Supporting Information Figure S5A,B). These results suggest that metformin produces a rapid onset of anxiolytic effects, *via* increasing the amplitude and frequency of mIPSCs in the dorsal hippocampal CA1 region.

Consistent with *in vitro* experiments, we found that metformin activated the AMPK-FoxO3a-GABARAP signalling pathway in the dorsal hippocampal CA1 region (Figure 7A). Additionally, the coimmunoprecipitation between $GABA_A$ receptor γ_2 subunit and GABARAP was increased by metformin (Figure 7B). Furthermore, intracranial injection of rats with

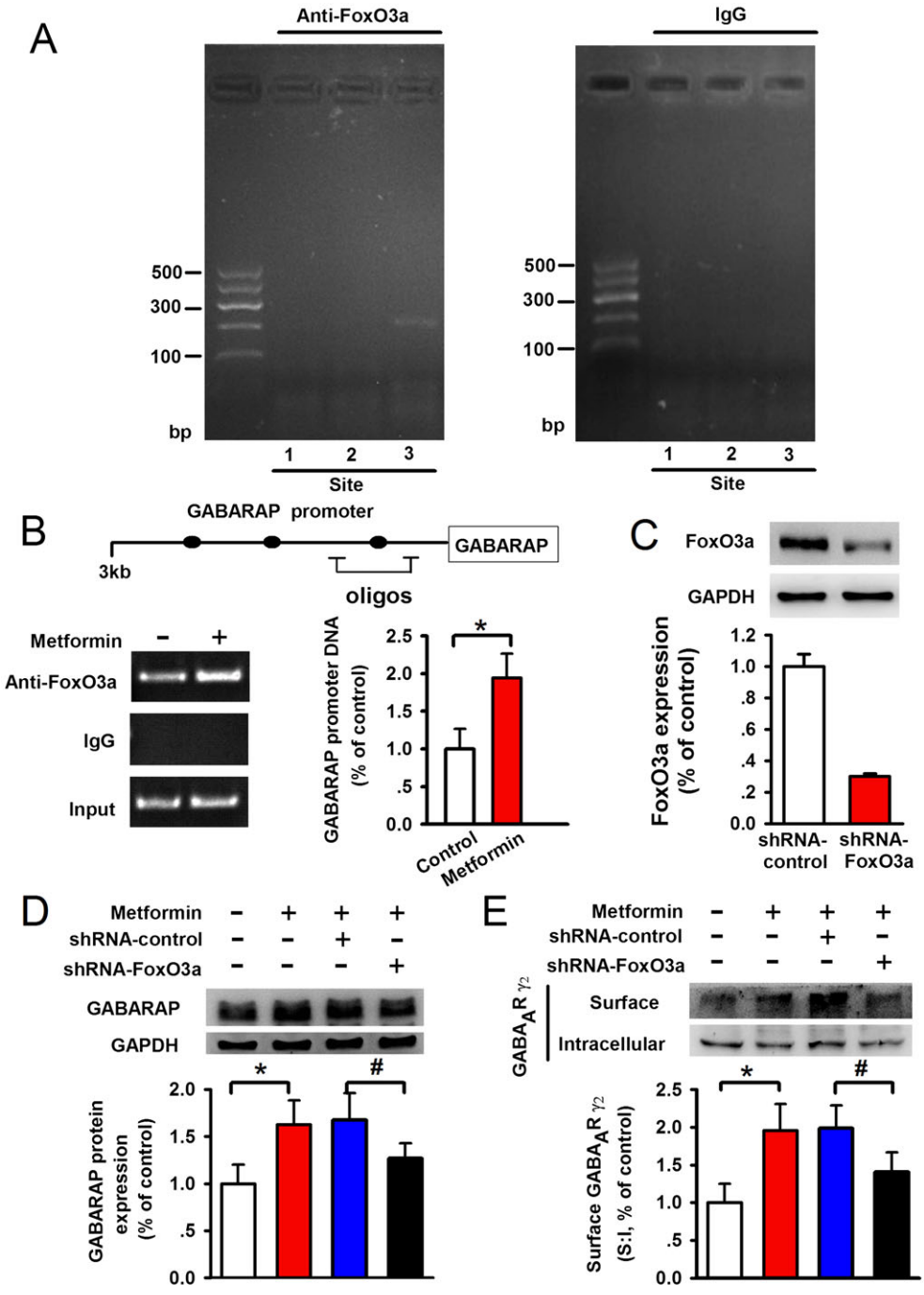

Figure 4

FoxO3a is involved in metformin-induced up-regulation of GABARAP expression and surface expression of $GABA_A$ receptors. (A–B) ChIP analyses indicated that metformin increased the binding of FoxO3a to the GABARAP promoter (n = 5 per group). (C) Hippocampal neurons transfected with shRNA-FoxO3a showed a decrease in the FoxO3a protein expression (n = 3 per group). (D–E) Western blot analysis revealing that shRNA-FoxO3a significantly prevented metformin-induced expression of GABARAP (D) and the surface expression of $GABA_A$ receptor γ_2 subunits (E) in neurons (n = 6 per group). Data are expressed as the means ± SEM., one-way ANOVA with Bonferonni *post hoc* test, $^*P < 0.05$, significantly different from control; $^{\#}P < 0.05$, significantly different from shRNA.

the TAT-conjugated peptide that designed to disrupt the GABARAP– $GABA_A$ receptor interaction in the dorsal hippocampal CA1 region effectively prevented the increase in centre duration (Figure 7C,D) and the amplitude and frequency of mIPSCs (Supporting Information Figure S6C,D) induced by metformin, without affecting the basal anxiety-like behaviours (Supporting Information Figure S6F,G), indicating that the anxiolytic-like effects of metformin require an increased

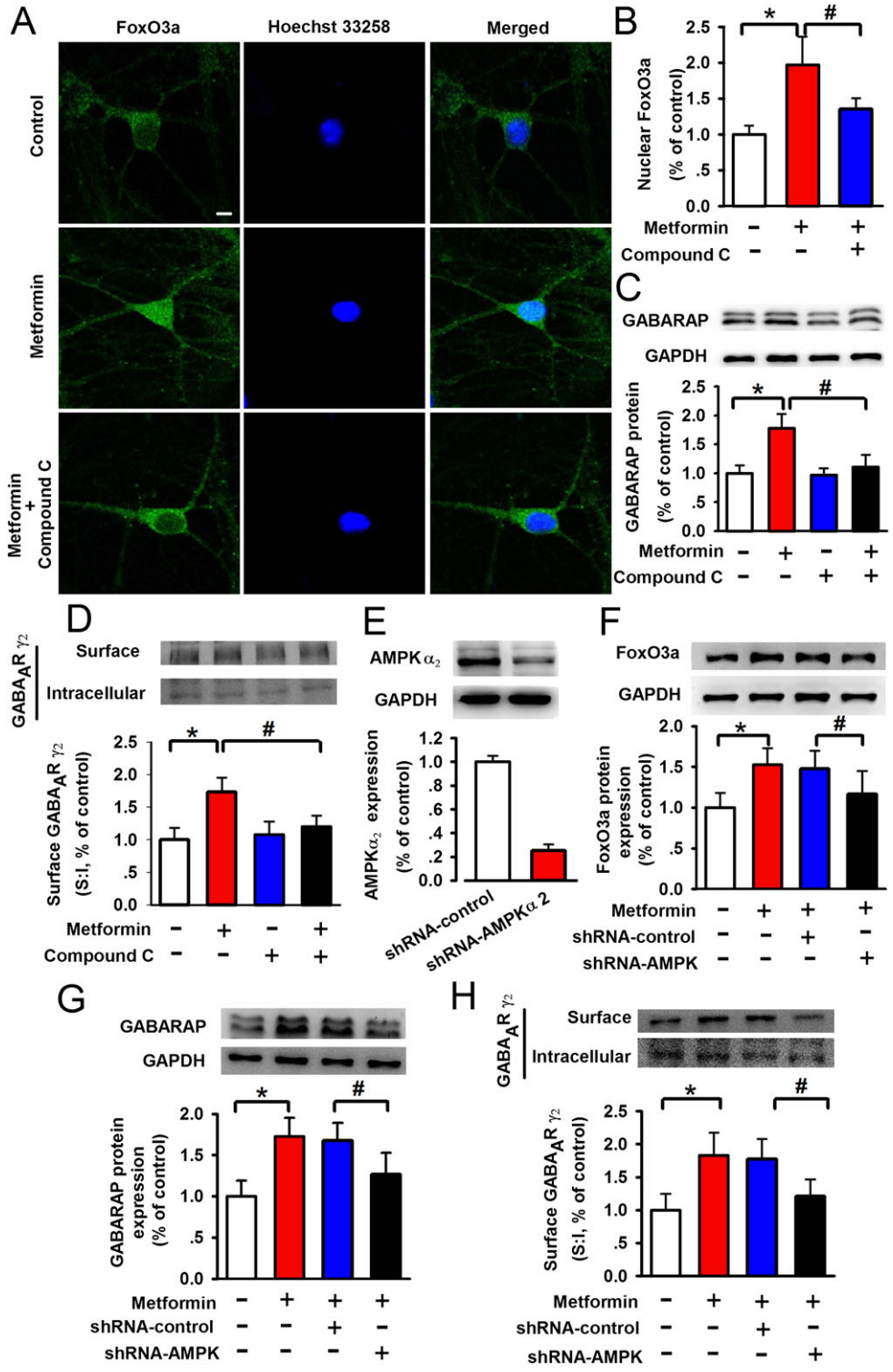

Figure 5

AMPK is involved in metformin-induced activation of FoxO3a signalling pathway. (A–B) Representative confocal images (A) and quantitative analysis (B) showing that Compound C (20 μM) inhibited metformin-induced (10 μM, 15 min) nuclear translocation of FoxO3a (green) ($n = 12$ per group). The merged image showing colocalization. Hoechst 33258 (blue). Scale bars: 20 μm. (C–D) Western blot showing that Compound C was sufficient to inhibit metformin-induced increase in the expression of GABARAP (C) and the surface expression of $GABA_A$ receptors (D). $n = 6$ per group. (E) Hippocampal neurons transfected with shRNA-AMPKα_2 displayed a decrease in protein expression ($n = 3$ per group). (F–H) Western blot analysis showing that shRNA-AMPKα_2 significantly prevented metformin-induced increase in the expression of FoxO3a (F), GABARAP (G) and the surface expression of γ_2-containing $GABA_A$ receptors (H). $n = 6$ per group. Data are expressed as the means ± SEM. One-way ANOVA with Bonferonni *post hoc* test. $^*P < 0.05$, significantly different from control, $^{\#}P < 0.05$, significantly different from Compound C or shRNA.

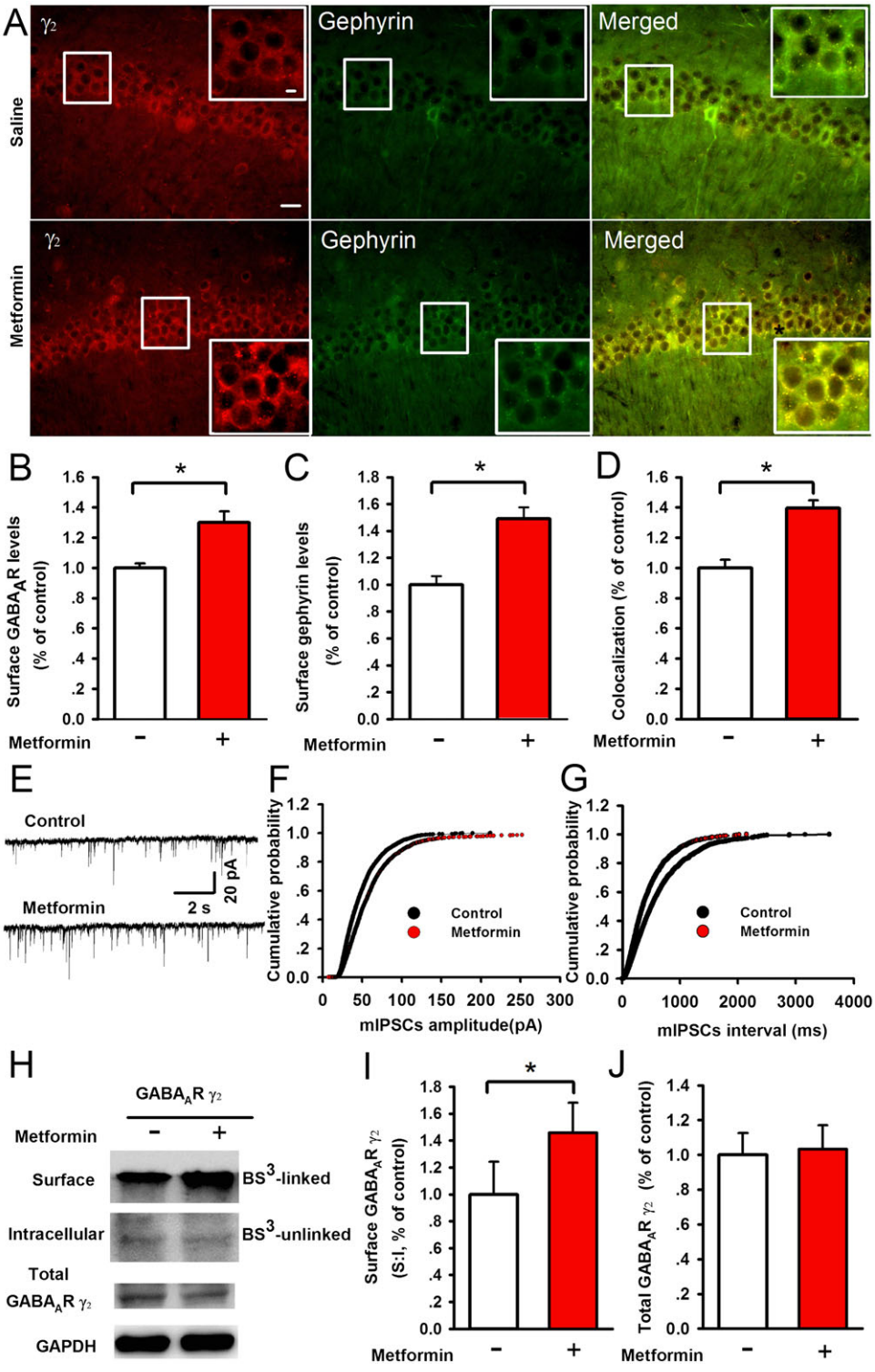

Figure 6

Metformin increases the surface expression of $GABA_AR$ γ_2 subunits and mIPSCs in rat hippocampal CA1 region *in vivo*. (A–C) Representative confocal images (A) and quantitative analysis showing that metformin (100 mg·kg^{-1}, i.p. 30 min) treatment markedly increased the surface intensity of γ_2-containing $GABA_A$ receptors (red, B), the receptor anchoring protein gephyrin (green, C) and the overlapping puncta (D) in the dorsal hippocampal CA1 region. $n = 5$ per group. Scale bars: 20 μm. (E) Representative traces of mIPSCs before (control) and after application of 10 μM metformin on hippocampal CA1 slice. (F–G) Representative cumulative plots of mIPSC amplitude (F) and inter-event interval (G). $n = 10$ per group. (H–J) Western blot analysis showing that metformin (100 mg·kg^{-1}, i.p. 30 min) treatment increased the surface expression of $GABA_A$ receptor γ_2 subunits (I) and unaffected the total $GABA_A$ receptor γ_2 subunits level (J) in the hippocampal CA1 region. $n = 5$ per group. Data are expressed as means ± SEM. *$P < 0.05$, significantly different from control group.

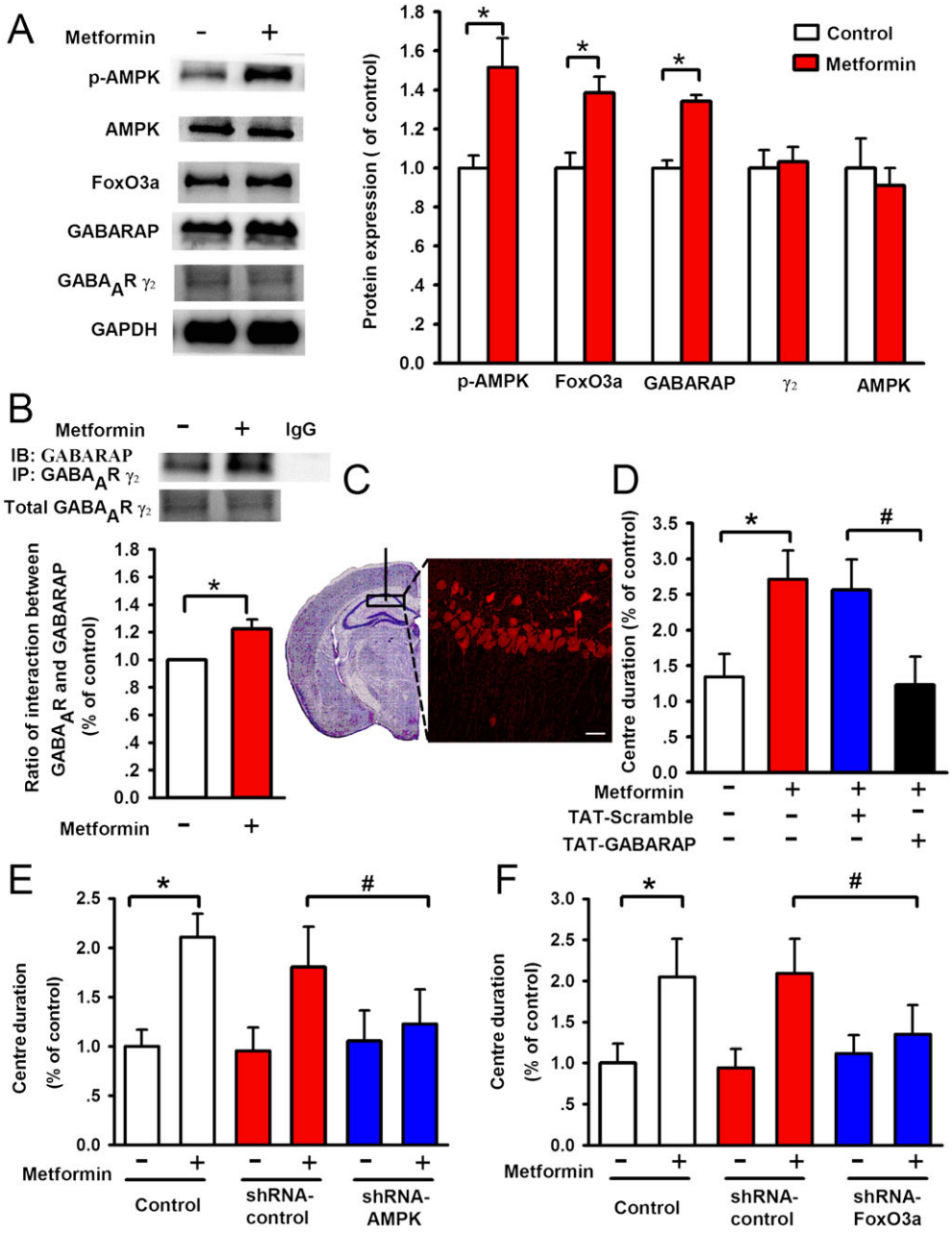

Figure 7

AMPK-FoxO3a-GABARAP pathway in the dorsal CA1 region is involved in the anxiolytic effect of metformin. (A) Western blot analysis showing that metformin (100 mg·kg^{-1}, i.p., 30 min) treatment significantly increased the expression of p-AMPK, FoxO3a, GABARAP ($n = 6$ per group). (B) Co-IP analysis showing that metformin increased binding of GABARAP to GABA$_A$ receptor γ_2 subunits without affecting the total expression of GABA$_A$ receptor γ_2 subunits ($n = 6$ per group). (C) Distribution of 5-TAMRA (red) fluorescent-labelled TAT-conjugated peptide in the dorsal hippocampal CA1 region. Scale bar: 50 μm. (D) In the OFT test, intracranial injection of TAT-conjugated peptide effectively prevented the increase in central duration induced by metformin ($n = 9$ per group). (E) Intracranial injection of shRNA-AMPKα_2 effectively decreased the central duration induced by metformin (control: $n = 9$ per group; shRNA-control: $n = 6$ per group; shRNA-AMPKα_2: $n = 8$ per group). (F) Intracranial injection of shRNA-FoxO3a effectively decreased the central duration induced by metformin (control: $n = 10$ per group; shRNA-control: $n = 7$ per group; shRNA-FoxO3a: $n = 10$ per group). Data are expressed as means ± SEM. One- or two-way ANOVA with Bonferonni *post hoc* test. $^*P < 0.05$, significantly different from control, $^{\#}P < 0.05$, significantly different from shRNA-control or TAT-scramble.

interaction between GABARAP and GABA$_A$ receptor γ_2 subunits. Moreover, intracranial injection of shRNA-AMPKα_2 (Figure 7E) and shRNA-FoxO3a (Figure 7F) prevented the metformin-induced anxiolytic-like behaviour in rats.

Discussion

The findings presented here demonstrate, for the first time, that the widely used antidiabetic drug metformin

displays a rapid onset of anxiolytic-like effects *via* up-regulation of the surface expression of $GABA_A$ receptors . AMPK plays a critical role in metformin-induced activation of FoxO3a transcriptional activity, leading to the increase in GABARAP expression, which is central to the membrane insertion of $GABA_A$ receptors. These results defined a distinct role of metformin in orchestration of the efficacy of GABAergic synaptic transmission and raise the possibility that metformin may be used as a new treatment for anxiety disorders.

Metformin exerted anxiolytic effect without tolerance by enhancing postsynaptic accumulation of $GABA_A$ receptors in the cell membrane

The therapeutic approaches to treat anxiety disorders in animal studies or in clinical trials have focused primarily on the activator of $GABA_A$ receptors, such as the benzodiazepines. These drugs are commonly prescribed with major clinical significance, but the development of tolerance restricts their clinical use and has encouraged a search for other mechanisms of anxiety disorder for several decades. Treatment with the $GABA_A$ receptor agonist muscimol in *Caenorhabditis elegans* resulted in selective removal of these receptors from synapses during adaptation to muscimol (Davis *et al*., 2010). A recent report indicates that in cultured hippocampal neurons, flurazepam exposure enhances degradation of $GABA_A$ receptors after their removal from the plasma membrane, leading to a reduction in inhibitory synapse size and number along with a decrease in the efficacy of synaptic inhibition (Jacob *et al*., 2012). The results provide a mechanism for initial adaptations after treatment with benzodiazepines. Here, we demonstrated that metformin potentiated inhibitory synaptic transmission *via* enhancing postsynaptic accumulation of $GABA_A$ receptors in the cell membrane, which was a mechanism different from that of the benzodiazepines. Meanwhile, our *in vivo* studies indicate that chronic treatment with metformin did not induce tolerance, in clear contrast to the classical anxiolytic, diazepam. This reduction in drug tolerance may be due to exocytosis of $GABA_A$ receptors, rather than the endocytosis and degradation of $GABA_A$ receptors induced by benzodiazepines (Jacob *et al*., 2012).

GABARAP mediates the anxiolytic effect of metformin by regulating the surface expression of $GABA_A$ receptors

Several studies have demonstrated that GABARAP enhances the surface expression of γ_2-containing $GABA_A$ receptors in both heterologous expression systems and cultured hippocampal neurons (Kawaguchi and Hirano, 2007; Marsden *et al*., 2007). However, the GABARAP knockout mouse is phenotypically normal and exhibits no effects on synaptic puncta of $GABA_A$ receptors, suggesting the possibility that GABARAP plays little role in maintaining basal surface levels of $GABA_A$ receptors but merely accelerates or promotes transportation of intracellular $GABA_A$ receptors to the plasma membrane (O'Sullivan *et al*., 2005). In addition, GABARAP has also been characterized as a mammalian homologue of the yeast Atg8 and critically involved in autophagy (Tanida *et al*., 2003; Weidberg *et al*., 2010). However, there is little endogenous GABARAP lipidation during autophagy in the rat brain, suggesting that GABARAP is involved in cellular functions other than autophagy in the brain (Koike *et al*., 2013). We found that GABARAP mainly facilitated the activity-dependent translocation of $GABA_A$ receptors from intracellular compartments to the somatodendritic plasma membrane in neuron. A TAT-conjugated peptide delivered to CA1 region further demonstrated that GABARAP may facilitates membrane insertion of $GABA_A$ receptors and thus lead to the anxiolytic effects of metformin. However, further study is required to determine whether the amygdala and other regions of hippocampus (such as CA3 and DG) that are involved in the regulation of anxiety, are also involved in the anxiolytic effect of metformin.

Metformin improves anxiety-like behaviour via *AMPK activation, which is independent of its glucose-lowering effects*

Metformin is a widely used drug for treatment of Type 2 diabetes. The maximum therapeutic dose of metformin in a 70 kg man is approximately 35 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ (Kinaan *et al*., 2015). Increasing evidence suggests that AMPK activation is required for inhibitory effect of metformin on glucose production and stimulates glucose uptake (Zhou *et al*., 2001; Abbasi *et al*., 2004). Our results showed that a sub-therapeutic dose of metformin (100 $mg{\cdot}kg^{-1}$, approximately 15.9 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) significantly reduced the anxiety-like behaviours in rats by activation of AMPK, independent of its glucose-lowering effects. Kuramoto and colleagues favoured the idea that AMPK activation was neuroprotective, which may be attributed to the increased function of $GABA_B$ receptors to reduce excitotoxicity and thereby promoted neuronal survival (Kuramoto *et al*., 2007). However, there is no evidence for a relationship between AMPK and $GABA_A$ receptor-mediated synaptic transmission. Here, our study revealed that metformin, an AMPK activator, robustly increased the membrane trafficking of $GABA_A$ receptors and facilitated inhibitory synaptic transmission in neurons, which provided a new and potential pharmacological approach to treat GABAergic deficits in mood disorders.

Metformin can improve both the primary anxiety disorder and the anxiety-like behaviour secondary to other disorders

Sarkaki *et al*. found that pretreatment with metformin for 2-weeks improved anxiety-like behaviour, following global cerebral ischaemia model, through increasing autophagy (Sarkaki *et al*., 2015). Meanwhile, metformin had an anxiolytic effect and improved sensorimotor scores after focal ischaemia in a diabetic rat model (Prakash *et al*., 2013). These results suggest that metformin could relieve anxiety-like behaviour secondary to cerebral ischaemia, with or without diabetes, which may be related to the neuroprotective effect of metformin on cerebral ischaemia. Whether or not metformin could relieve the primary anxiety disorder remains largely unknown. Interestingly, in our study, we found that metformin (100 $mg{\cdot}kg^{-1}$, i.p., 30 min) could reduce anxiety-like behaviour in both OFT and EPM assays, suggesting that metformin could rapidly improve the primary anxiety disorder. In previous studies, the dose of metformin

(200 mg·kg^{-1}·mL^{-1} i.p. for 14 days or 300 mg·kg^{-1}·day^{-1} in drinking water for 5 days) used to relieve anxiety-like behaviour secondary to the cerebral ischaemia or diabetes was higher than that in our study (100 mg·kg^{-1}, i.p. for 30 min) (Prakash *et al.*, 2013; Sarkaki *et al.*, 2015). This discrepancy may be due to the difference between primary diseases. The anxiolytic effects may only be achieved when the underlying diseases are improved, indicating that the neuroprotective effect of metformin is responsible for the behavioural and emotional revival following global cerebral ischaemia. However, in the present study, the rapid relief, induced by metformin, of anxiety was attributed to the increase in the trafficking of $GABA_A$ receptors to the cell membrane, thus facilitating inhibitory synaptic transmission, which is a novel mechanism of the action of metformin.

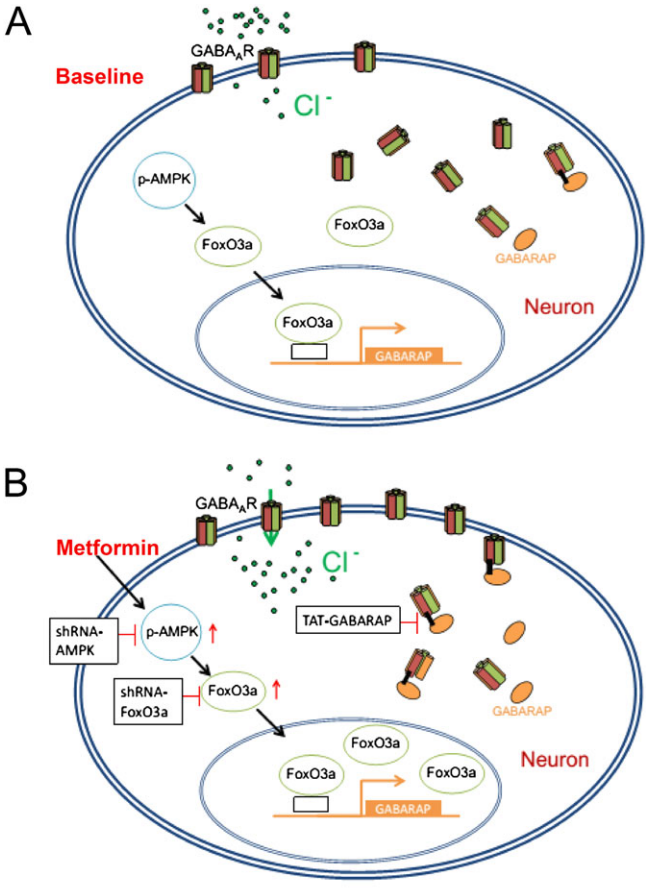

Figure 8

Mechanisms of metformin-induced membrane trafficking $GABA_A$Rs in hippocampal neurons. (A) Under the baseline condition, AMPK is involved in FoxO3a activation that directly binds to the promoter region and induces the expression of GABARAP; endogenous GABARAP interacts with $GABA_A$Rs and contributes to the regulation of membrane insertion of $GABA_A$Rs. (B) When treated with metformin, AMPK is activated rapidly and then increases FoxO3a expression and nuclear translocation, which leads to endogenous GABARAP expression and facilitates its interaction with $GABA_A$Rs. This will result in an increase in surface expression of $GABA_A$Rs and facilitate miniature inhibitory currents.

The high dosage of metformin may produce sedative-like effect

In our study, high dosage of metformin prominently reduced the locomotor activity during the OFT, suggesting that metformin may have sedative properties at higher doses. The sedative and anxiolytic-like actions of diazepam are mainly mediated by α_1 (McKernan *et al.*, 2000) and α_2 (Low *et al.*, 2000) containing $GABA_A$ receptors respectively. In addition to the γ_2 subunit, we also found that the anxiolytic dose of metformin increased the expression of the α_1 and α_2 subunit in the plasma membrane, which supported the anxiolytic effect of metformin, as well as explaining the effects of high dose metformin on behaviours during OFT. In order to guide clinical use of metformin to treat anxiety disorders in the future, further studies will be necessary to define the detailed mechanism of the sedative effect of metformin.

In conclusion, we have been able to show that one of the most widely used oral hypoglycaemic drugs, metformin, can promote the membrane insertion of $GABA_A$ receptors in neurons *via* activating the AMPK-FoxO3a-GABARAP signalling pathway, thus enhancing neurotransmission at inhibitory synapses and, finally, producing a rapid anxiolytic effect, without tolerance (Figure 8). Our results raise the possibility that this decades-old antidiabetic drug could be used to treat anxiety disorders. The safety of metformin has long been established in humans and, most importantly, our results may provide an alternative to diazepam, the most frequently used anxiolytic agent in clinical practice. Because GABAergic deficits are essential to the aetiology of neuropsychiatric disorders, such as major depressive disorder (Luscher *et al.*, 2011b), bipolar disorder (Craddock *et al.*, 2010) and schizophrenia (Charych *et al.*, 2009), our work may provide a promising new use for an old drug, metformin, in the treatment of neuropsychiatric disorders *via* regulation of $GABA_A$ receptor trafficking to the neuronal cell membrane.

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Author contributions

J.G.C. and F.W. designed the research. J.F. and D.L. performed the culture of primary hippocampal neuron, western blotting and immunofluorescence. J.G.H. and W.W.Z. measured the metformin content in rats. J.F. and H.S.C. carried out electrophysiological recording. D.L., J.F.X. and J.F. conducted the behavioural tests and analysis. D.L. measured the level of blood glucose in rats. D.L., H.S.C. and J.F. performed

stereotaxic microinjection. F.W. and J.F. analysed the data and wrote the paper. All authors contributed to analysis and discussion of the results.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Abbasi F, Chu JW, McLaughlin T, Lamendola C, Leary ET, Reaven GM (2004). Effect of metformin treatment on multiple cardiovascular disease risk factors in patients with type 2 diabetes mellitus. Metabolism 53: 159–164.

Adams W, van den Buuse M (2011). Hippocampal serotonin depletion facilitates the enhancement of prepulse inhibition by risperidone: possible role of 5-HT(2C) receptors in the dorsal hippocampus. Neuropharmacology 61: 458–467.

Alexander SPH, Peters JA, Kelly E, Marrion NV, Faccenda E, Harding SD *et al.* (2017a). The Concise Guide to PHARMACOLOGY 2017/18: Ligand-gated ion channels. Br J Pharmacol 174: S130–S159.

Alexander SPH, Fabbro D, Kelly E, Marrion NV, Peters JA, Faccenda E *et al.* (2017b). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. Br J Pharmacol 174: S272–S359.

Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA *et al.* (2017c). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. Br J Pharmacol 174: S17–S129.

Boudreau AC, Wolf ME (2005). Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J Neurosci 25: 9144–9151.

Brynildsen JK, Lee BG, Perron IJ, Jin S, Kim SF, Blendy JA (2018). Activation of AMPK by metformin improves withdrawal signs precipitated by nicotine withdrawal. Proc Natl Acad Sci U S A 115: 4282–4287.

Buller R, Legrand V (2001). Novel treatments for anxiety and depression: hurdles in bringing them to the market. Drug Discov Today 6: 1220–1230.

Charych EI, Liu F, Moss SJ, Brandon NJ (2009). GABA(A) receptors and their associated proteins: implications in the etiology and treatment of schizophrenia and related disorders. Neuropharmacology 57: 481–495.

Chemali JJ, Van Dort CJ, Brown EN, Solt K (2012). Active emergence from propofol general anesthesia is induced by methylphenidate. Anesthesiology 116: 998–1005.

Chen ZW, Olsen RW (2007). GABAA receptor associated proteins: a key factor regulating GABAA receptor function. J Neurochem 100: 279–294.

Chen ZW, Chang CS, Leil TA, Olcese R, Olsen RW (2005). GABAA receptor-associated protein regulates GABAA receptor cell-surface number in Xenopus laevis oocytes. Mol Pharmacol 68: 152–159.

Chen T, Yang YJ, Li YK, Liu J, Wu PF, Wang F *et al.* (2016). Chronic administration tetrahydroxystilbene glucoside promotes hippocampal memory and synaptic plasticity and activates ERKs, CaMKII and SIRT1/miR-134 in vivo. J Ethnopharmacol 190: 74–82.

Chiacchiera F, Simone C (2010). The AMPK-FoxO3A axis as a target for cancer treatment. Cell Cycle 9: 1091–1096.

Craddock N, Jones L, Jones IR, Kirov G, Green EK, Grozeva D *et al.* (2010). Strong genetic evidence for a selective influence of GABAA receptors on a component of the bipolar disorder phenotype. Mol Psychiatry 15: 146–153.

Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Giembycz MA *et al.* (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Brit J Pharmacol 175: 987–993.

Davis KM, Sturt BL, Friedmann AJ, Richmond JE, Bessereau JL, Grant BD *et al.* (2010). Regulated lysosomal trafficking as a mechanism for regulating GABAA receptor abundance at synapses in Caenorhabditis elegans. Mol Cell Neurosci 44: 307–317.

Edwards LE, Mezuk B (2012). Anxiety and risk of type 2 diabetes: evidence from the Baltimore Epidemiologic Catchment Area Study. J Psychosom Res 73: 418–423.

Everitt AB, Luu T, Cromer B, Tierney ML, Birnir B, Olsen RW*et al.* (2004). Conductance of recombinant GABA(A) channels is increased in cells co-expressing GABA(A) receptor-associated protein. J Biol Chem 279: 21701–21706.

Furuyama T, Nakazawa T, Nakano I, Mori N (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J 349: 629–634.

Gantois I, Khoutorsky A, Popic J, Aguilar-Valles A, Freemantle E, Cao R *et al.* (2017). Metformin ameliorates core deficits in a mouse model of fragile X syndrome. Nat Med 23: 674–677.

Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D *et al.* (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 17: 1646–1656.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S *et al.* (2018). The IUPHAR/BPS guide to pharmacology in 2018: updates and expansion to encompass the new guide to immunopharmacology. Nucl Acids Res 46: D1091–D1106.

Hou X, Song J, Li XN, Zhang L, Wang X, Chen L *et al.* (2010). Metformin reduces intracellular reactive oxygen species levels by upregulating expression of the antioxidant thioredoxin via the AMPK-FOXO3 pathway. Biochem Biophys Res Commun 396: 199–205.

Jacob TC, Michels G, Silayeva L, Haydon J, Succol F, Moss SJ (2012). Benzodiazepine treatment induces subtype-specific changes in GABA(A) receptor trafficking and decreases synaptic inhibition. Proc Natl Acad Sci U S A 109: 18595–18600.

Jimenez JC, Su K, Goldberg AR, Luna VM, Biane JS, Ordek G *et al.* (2018). Anxiety cells in a hippocampal-hypothalamic circuit. Neuron 97: 670–683 e676.

Kawaguchi SY, Hirano T (2007). Sustained structural change of GABA(A) receptor-associated protein underlies long-term potentiation at inhibitory synapses on a cerebellar Purkinje neuron. J Neurosci 27: 6788–6799.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kim CS, Chang PY, Johnston D (2012). Enhancement of dorsal hippocampal activity by knockdown of HCN1 channels leads to anxiolytic- and antidepressant-like behaviors. Neuron 75: 503–516.

Kinaan M, Ding H, Triggle CR (2015). Metformin: an old drug for the treatment of diabetes but a new drug for the protection of the endothelium. Med Princ Pract 24: 401–415.

Koike M, Tanida I, Nanao T, Tada N, Iwata J, Ueno T*et al*. (2013). Enrichment of GABARAP relative to LC3 in the axonal initial segments of neurons. PLoS One 8: e63568.

Kuramoto N, Wilkins ME, Fairfax BP, Revilla-Sanchez R, Terunuma M, Tamaki K *et al*. (2007). Phospho-dependent functional modulation of GABA(B) receptors by the metabolic sensor AMP-dependent protein kinase. Neuron 53: 233–247.

Leil TA, Chen ZW, Chang CS, Olsen RW (2004). GABAA receptor-associated protein traffics GABAA receptors to the plasma membrane in neurons. J Neurosci 24: 11429–11438.

Lewko J, Zarzycki W, Krajewska-Kulak E (2012). Relationship between the occurrence of symptoms of anxiety and depression, quality of life, and level of acceptance of illness in patients with type 2 diabetes. Saudi Med J 33: 887–894.

Li MX, Zheng HL, Luo Y, He JG, Wang W, Han J *et al*. (2018). Gene deficiency and pharmacological inhibition of caspase-1 confers resilience to chronic social defeat stress via regulating the stability of surface AMPARs. Mol Psychiatry 23: 556–568.

Liu GX, Cai GQ, Cai YQ, Sheng ZJ, Jiang J, Mei Z *et al*. (2007). Reduced anxiety and depression-like behaviors in mice lacking GABA transporter subtype 1. Neuropsychopharmacology 32: 1531–1539.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA *et al*. (2000). Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290: 131–134.

Lu HF, Wu PF, Yang YJ, Xiao W, Fan J, Liu J *et al*. (2014). Interactions between N-ethylmaleimide-sensitive factor and GluR2 in the nucleus accumbens contribute to the expression of locomotor sensitization to cocaine. J Neurosci 34: 3493–3508.

Luscher B, Fuchs T, Kilpatrick CL (2011a). GABAA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron 70: 385–409.

Luscher B, Shen Q, Sahir N (2011b). The GABAergic deficit hypothesis of major depressive disorder. Mol Psychiatry 16: 383–406.

Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P *et al*. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 6: 458–471.

Marsden KC, Beattie JB, Friedenthal J, Carroll RC (2007). NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of GABA(A) receptors. J Neurosci 27: 14326–14337.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR *et al*. (2000). Sedative but not anxiolytic properties of benzodiazepines ave mediated by the GABA(A) receptor α(1) subtype. Nat Neurosci 3: 587–592.

Modol L, Darbra S, Pallares M (2011). Neurosteroids infusion into the CA1 hippocampal region on exploration, anxiety-like behaviour and aversive learning. Behav Brain Res 222: 223–229.

Naseri MH, Hesami-Tackallou S, Torabi-Nami M, Zarrindast MR, Nasehi M (2014). Involvement of the CA1 GABAA receptors in MK-801-induced anxiolytic-like effects: an isobologram analysis. Behav Pharmacol 25: 197–205.

Obsil T, Obsilova V (2011). Structural basis for DNA recognition by FOXO proteins. Biochim Biophys Acta 1813: 1946–1953.

O'Sullivan GA, Kneussel M, Elazar Z, Betz H (2005). GABARAP is not essential for GABA receptor targeting to the synapse. Eur J Neurosci 22: 2644–2648.

Prakash R, Li W, Qu Z, Johnson MA, Fagan SC, Ergul A (2013). Vascularization pattern after ischemic stroke is different in control versus diabetic rats: relevance to stroke recovery. Stroke 44: 2875–2882.

Rannals MD, Kapur J (2011). Homeostatic strengthening of inhibitory synapses is mediated by the accumulation of GABA(A) receptors. J Neurosci 31: 17701–17712.

Sarkaki A, Farbood Y, Badavi M, Khalaj L, Khodagholi F, Ashabi G (2015). Metformin improves anxiety-like behaviors through AMPK-dependent regulation of autophagy following transient forebrain ischemia. Metab Brain Dis 30: 1139–1150.

Sato A, Sunayama J, Okada M, Watanabe E, Seino S, Shibuya K *et al*. (2012). Glioma-initiating cell elimination by metformin activation of FOXO3 via AMPK. Stem Cells Transl Med 1: 811–824.

Smith KS, Rudolph U (2012). Anxiety and depression: mouse genetics and pharmacological approaches to the role of GABA(A) receptor subtypes. Neuropharmacology 62: 54–62.

Tanida I, Komatsu M, Ueno T, Kominami E (2003). GATE-16 and GABARAP are authentic modifiers mediated by Apg7 and Apg3. Biochem Biophys Res Commun 300: 637–644.

Tasan RO, Bukovac A, Peterschmitt YN, Sartori SB, Landgraf R, Singewald N *et al*. (2011). Altered GABA transmission in a mouse model of increased trait anxiety. Neuroscience 183: 71–80.

Tovilla-Zarate C, Juarez-Rojop I, Peralta Jimenez Y, Jimenez MA, Vazquez S, Bermudez-Ocana D *et al*. (2012). Prevalence of anxiety and depression among outpatients with type 2 diabetes in the Mexican population. PLoS One 7: e36887.

Tunc-Ozcan E, Wert SL, Lim PH, Ferreira A, Redei EE (2017). Hippocampus-dependent memory and allele-specific gene expression in adult offspring of alcohol-consuming dams after neonatal treatment with thyroxin or metformin. Mol Psychiatry https://doi.org/10.1038/mp.2017.129 23: 1643–1651.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999). GABA(A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 397: 69–72.

Wang W, Wang F, Yang YJ, Hu ZL, Long LH, Fu H *et al*. (2011). The flavonoid baicalein promotes NMDA receptor-dependent long-term potentiation and enhances memory. Br J Pharmacol 162: 1364–1379.

Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z (2010). LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 29: 1792–1802.

Wells CE, Amos DP, Jeewajee A, Douchamps V, Rodgers J, O'Keefe J *et al*. (2013). Novelty and anxiolytic drugs dissociate two components of hippocampal theta in behaving rats. J Neurosci 33: 8650–8667.

Zarrindast MR, Nasehi M, Pournaghshband M, Yekta BG (2012). Dopaminergic system in CA1 modulates MK-801 induced anxiolytic-like responses. Pharmacol Biochem Behav 103: 102–110.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J *et al*. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108: 1167–1174.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

https://doi.org/10.1111/bph.14519

Figure S1 The tolerance is observed in diazepam-treated rats after 14-day administration. The rats were treated with vehicle or diazepam (1 mg·kg^{-1}, i.p.) for single (30 min) or successive 14 days injection and the anxiety-like behavior was evaluated in the open field test. Data are expressed as the means ± s.e.m. *$P < 0.05$. $^{\#}P < 0.05$, one-way ANOVA with Bonferonni *post hoc* test.

Figure S2 The effect of metformin on blood glucose, electroencephalogram, working memory and fear conditioning of rats. (A-B) There are no significant differences in the blood glucose in saline and metformin-treated (100 mg·kg^{-1}, i.p.) rats for single or successive 14 days injection ($n = 10$). (C) Representative EEG trace before and after metformin treated, and bar plot summary showing the effect of metformin on electroencephalogram (EEG) of rats ($n = 5$ per group). (D) The effect of Single administration with metformin (100 mg·kg^{-1}, i.p.) on working memory was evaluated in T-maze test ($n = 10$ per group). (E) Rats treated with metformin (100 mg·kg^{-1}, i.p, 30 min) were performed fear conditioning training, 24 h later, contextual- and cue-induced fear memory were test ($n = 10$ per group). Data are expressed as the means ± s.e.m. *$P < 0.05$ compared with control.

Figure S3 The mean concentration of metformin after acute intraperitoneal administration (100 mg·kg^{-1}) in plasma (A) and cerebrospinal fluid (B). n=6.

Figure S4 Metformin has no effect on excitatory glutamate receptors membrane trafficking in cultured neurons. (A) A representative β-actin and γ2 subunit of $GABA_AR$ immunoblot from hippocampal neurons treated with bis (sulfosuccinimidyl) suberate (BS^3) or vehicle control (Con). (B) Western blot analysis showing no significant difference in surface level of GluA1-containing AMPARs between two groups. Data are expressed as means ± s.e.m. n=3.

Figure S5 The effect of metformin on the expression of GABA receptor and glutamate receptor subunits in dorsal hippocampal CA1 region. (A) Metformin induced an increase in protein abundance of the $GABA_AR$ α_1 and α_2 subunit and had no effects on total amount of $GABA_AR$ α_1 and α_2 subunit. (B) Metformin did not affect the surface and total expression of $GABA_{B2}$ and glutamate receptor subunits. Data are expressed as means ± s.e.m. n=5. *$P < 0.05$ compared with control.

Figure S6 The effect of GABARAP inhibitory peptide on mIPSCs induced by metformin and the basal inhibitory transmission. (A-B) Bar plot summary showing metformin induces a significant increase the amplitude and frequency of mIPSCs in the dorsal hippocampal CA1 region ($n = 10$ per group). (C-D) The GABARAP inhibitory peptide prevents the increase mIPSCs induced by metformin in the dorsal hippocampal CA1 region ($n = 4$ per group). (E-F) The GABARAP inhibitory peptide did not affect the basal surface expression of $GABA_AR$ in cultured neurons (E $n = 3$ per group), the basal anxiety-like behavior (F, $n = 9$ per group) and locomotor activity (G, $n = 9$ per group) in the open field test. Data are expressed as the means ± s.e.m. *$P < 0.05$ compared with control.

Table S1 The numbers (n) of rats for each group in *in vivo* study.

Table S2 The numbers (n) for each group in *in vitro* study.

Table S3 Pharmacokinetic parameters of metformin (100 mg·kg^{-1}, i.p.) in rats.